ONTOGENY AND BIOLOGICAL FUNCTION OF EPITHELIAL CELLS IN THE CHICKEN YOLK SAC AND SMALL INTESTINE

by

Haihan Zhang

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Eric Wong, Chair
Sally Johnson
Elizabeth Gilbert
Benjamin Corl

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ABSTRACT

(FOR GENERAL AUDIENCE)

The chicken yolk sac and small intestine are connected to each other and share many biological similarities. Both chicken small intestinal and yolk sac epithelia play critical roles for nutrient absorption and immune defense. In this dissertation, the mRNA for nutrient transporters such as the peptide transporter, PepT1 and the sodium-glucose co-transporter, SGLT1 were found to be expressed by absorptive epithelial cells in both the yolk sac and small intestine. Additionally, both intestinal and yolk sac epithelial cells expressed avian beta defensins (AvBDs), which are important chicken host defense peptides. In the small intestine, there are a number of differentiated cell types that originate from stem cells in the crypt that express the known mammalian stem cell markers, Olfm4 and Lgr5 mRNA. However, in the chicken yolk sac, only the stem cell marker Lgr5 mRNA was expressed by endothelial cells. In summary, the yolk sac epithelial cells are responsible for the absorptive and immune functions for the embryonic stage. The chicken small intestinal epithelial cells are derived from the intestinal stem cells in the crypts. These epithelial cells have different cell types, which function to absorb nutrients and secrete antimicrobial peptides.

Ontogeny and biological function of epithelial cells in the chicken yolk sac and small intestine

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ABSTRACT

The chicken yolk sac and small intestine are connected through the yolk stalk and share many biological similarities. During the embryonic stage, the extra-embryonic yolk sac helps the embryo to absorb nutrients primarily in the last two weeks of incubation. The chicken yolk sac physically moves yolk contents from the yolk sac to the small intestine at the end of embryogenesis. This is the time when the small intestine replaces the yolk sac in assimilating nutrients for the embryo and later for the posthatch chicken. Additionally, both chicken small intestinal epithelia and the yolk sac secrete beta defensins for promoting intestinal health. Since there are heterogeneous cell types along the mammalian intestinal villus, which are derived from the intestinal stem cells in the crypts, we investigated if cells of the chicken yolk sac and small intestine have the same ontogeny as mammalian intestinal epithelial cells. In this dissertation, we mainly focused on the spatial expression of nutrient transporters (PepT1 and SGLT1), intestinal stem cell markers (Lgr5 and Olfm4), and avian beta defensins in the chicken yolk sac and small intestine during the embryonic and early posthatch stages. RNAscope *in situ* hybridization was used to identify the distribution of cells expressing PepT1 mRNA in both the chicken yolk sac and small intestine. PepT1 mRNA was found to be expressed by epithelial cells in both the yolk sac and small intestine. In the yolk sac, PepT1 mRNA was uniformly distributed in each

endodermal epithelial cell along the villus-like structure. The pattern of PepT1 mRNA expression observed in the chicken yolk sac during the last 10 days of incubation revealed that PepT1 mRNA was increased from e11 to e13, and decreased from e15 to day of hatch. The peak of PepT1 mRNA expression was between e13 and e15, when the yolk sac reaches maximum absorptive area and the growth of the chicken embryo is at its fastest rate. However, the expression of PepT1 mRNA in the intestine was only detected in columnar enterocytes along the villus and not in goblet cells or cells in the crypts. The immunofluorescence assay confirmed that PepT1 protein was located at the brush border membrane of the enterocytes and that protein expression of PepT1 was restricted to the intestinal epithelial cells from approximately the middle to the tip of the villus. In order to identify intestinal stem cells, we used the known mammalian stem cell markers, Lgr5 and Olfm4. Both Lgr5 and Olfm4 are specifically expressed by cells in the chicken intestinal crypts, suggesting that they can be used as biomarkers for chicken intestinal stem cells. Dual labelling of PepT1 and Olfm4 mRNA on the same chicken intestinal sample revealed that there was a gap between PepT1-expressing enterocytes and Olfm4-expressing intestinal stem cells. The cells in this gap were presumably transit amplifying (TA) cells. Additionally, we also found that the TA cell zone along the intestinal villus was reduced during chicken growth. This TA cell population could be clearly detected at day of hatch and d1 posthatch but not later. The expression of SGLT1 mRNA was localized to yolk sac endodermal epithelial cells and showed a sharp increase at the end of incubation. This increase of SGLT1 mRNA coincided with the increase in glucose in the yolk, indicating that the chicken embryo needs glucose as energy for hatching. The mRNA expression profiles of various avian beta defensins have been examined by qPCR and *in situ* hybridization to investigate the immune function of the yolk sac and small intestine. We found that AvBD10 mRNA showed the highest

expression level in the yolk sac and was expressed predominantly in the yolk sac endodermal epithelial cells. Additionally, the expression of AvBD10 mRNA showed a development-specific pattern, which increased from e9 to e11, and decreased from e13 towards day of hatch. The expression patterns of AvBD1, 2, and 7 mRNA were similar to each other. These three genes were found to be expressed by chicken heterophils distributed in the yolk sac blood islands and small intestinal blood vessels. Only a subset of heterophils, which might be activated, were able to express AvBD1, 2, and 7 mRNA. In the intestine, the expression of AvBD10 mRNA was localized to cells along the villus at e19 and day of hatch, but later to only a few cells located above the intestinal crypts. In summary, the endodermal epithelial cells are responsible for the absorptive and immune functions of the chicken yolk sac. The yolk sac mesoderm is critical for embryonic hematopoiesis and innate immunity. The chicken small intestinal epithelial cells are derived from the intestinal stem cells in the crypts. These epithelial cells have different cell types, which are functioning to absorb nutrients and secrete antimicrobial peptides.

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Chapter I

Introduction

In the last decade, artificial selection has made a major contribution to the progress of broiler chicken performance. The age for broiler chickens sent to market has declined to 47 days and the slaughter weight of the birds at marketing age has increased from 1.2kg to 2.6kg. Even though the slaughter weight of broilers has been slowly increasing, the marketing age has stayed the same over the last 20 years (Figure 1.1). Limited outcomes from posthatch selection has led to the transition of broiler research to the prehatch and early posthatch stage, which are the critical periods for chicken development and growth. Chicken embryonic development is 21 days and the nutrients that support this process are mainly from the egg yolk and albumen. The chicken is a precocial animal, which can adapt to environmental changes like feed transition from egg nutrients to artificial or natural feed diets after hatch. During this time period, the newly hatched chicks are also experiencing a change in the nutrient absorptive organ from the yolk sac to the small intestine. The chicken yolk sac is an extra-embryonic tissue, which advances around embryonic day 3 and gradually covers the yolk content during chicken embryo development. During the last two weeks before hatch, when the chicken embryo grows about 90% of its birth weight, many nutrient transporters in the yolk sac are highly expressed, indicating the high capacity of the yolk sac to absorb nutrients for embryonic development. The chicken small intestine begins to develop approximately during the early stage of embryonic development (embryonic day 5) and is able to absorb nutrients at the last few days of incubation. The small intestine plays an essential role for the posthatch chicken to assimilate nutrients from feed. Thus, the last two weeks of the prehatch stage and first few weeks of the posthatch stage are quite critical for understanding chicken nutritional development and physiological changes.

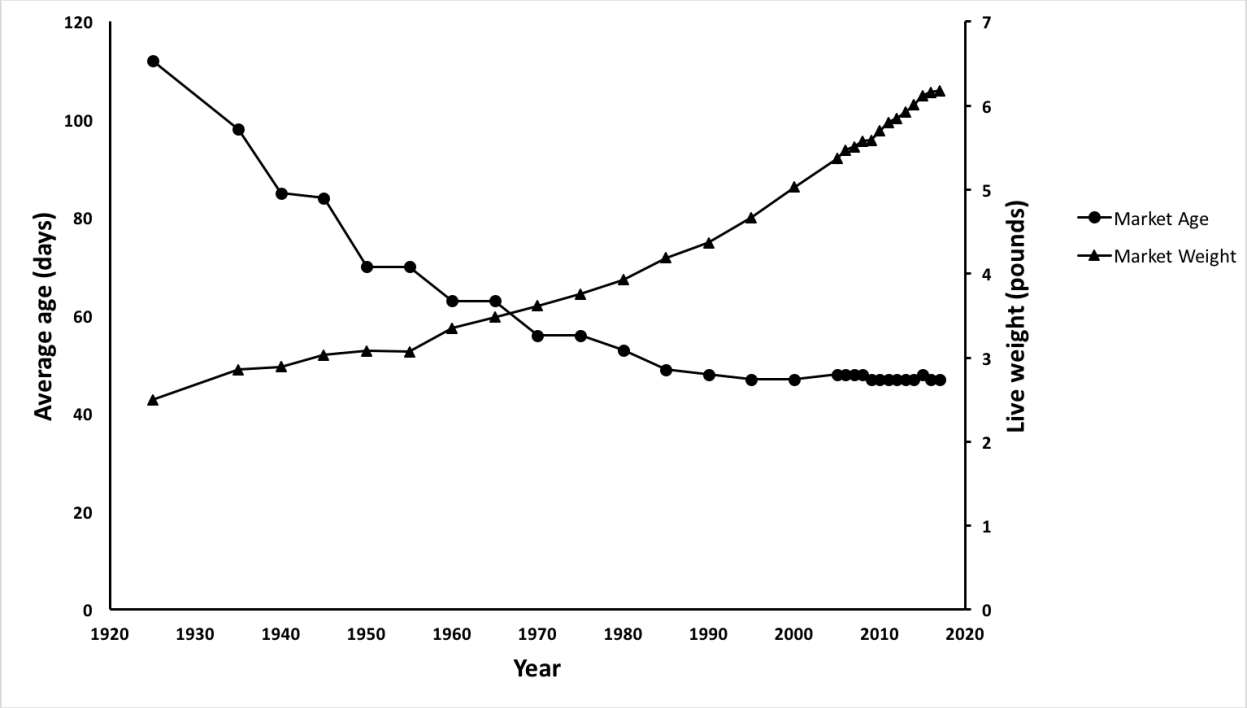

Figure 1.1 The change of broiler chicken market age and weight from 1925 to 2017. (Graph was made by H. Zhang and data were obtained from the National Chicken Council. **https://www.nationalchickencouncil.org**)

In this dissertation, the information presented in this literature review mainly focuses on comparing physiological and nutritional roles of the chicken yolk sac and small intestine. Some basic introduction to chicken embryo development and nutrient transporter functions will be covered as well as molecular cell biology research on the development of small intestine and yolk sac.

Chapter II

Literature Review

Development of the chicken embryo

The beginning of embryonic development traces back to the initiation of embryo fertilization that occurs at the infundibulum of the oviduct of the hen. After fertilization, the embryo travels down through the oviduct to form an intact fertilized egg. The magnum of the oviduct is a site with abundant glandular cells where materials forming the egg albumen are produced. The inner and outer shell membranes of the germ egg are synthesized at the isthmus of the oviduct. The formation of the egg shell occurs in the uterus. The complete procedure from ovulation to oviposition normally takes around 24 hours, while the embryo has been developing.

The entire period of chicken (*Gallus gallus*) embryogenesis is generally 21 days. Thus, a simple way to divide this incubation period is to separate it by days. However, the progression of embryo development is not an event strictly and uniformly set by chronological age. In fact, the embryo could make numerous changes during a particular time period but only a few changes during another time period. In order to distinguish apparent physiological and morphological changes during embryogenesis, Hamburger and Hamilton (1951) developed a standard, which is often called HH criterion, to describe the different phases of chicken embryonic development. They separated chicken embryogenesis into 46 stages and provided descriptions of apparent and essential changes of the embryo combined with corresponding pictures, which illustrate each developmental stage. However, one shortcoming of this strategy is that it did not take into account the morphological changes happening to extra-embryonic tissues, which are critical for chicken embryo development. In addition, the excessive divisions of embryonic development

periods may cause redundancy. For example, when looking at only nutritional development of the chicken embryo, it is better to follow a simple understanding of embryonic development, which regards the first third of incubation time as embryo establishment, the second third of incubation as embryo completion, and the last third period of incubation as preparation for emergence (Moran, 2007).

Composition and development of the chicken yolk sac

Compared with mammals and rodents, avian species develop their embryos separate from the mother. Thus, the embryo is only able to use nutrients deposited in the egg to support its growth and development. The yolk and albumen are the major nutrient sources for the developing chicken embryo. The yolk is made up of macromolecules synthesized and secreted by the maternal liver (Schneider *et al.*, 1998). The chicken yolk consists of approximately 50% water, 33% lipid, 15% protein and 1% carbohydrates. The albumen makes up 65% to 75% of the whole egg content and contains 12% protein and 88% water (Romanoff, 1960; Shenstone, 1968). These nutrients, which make up the entire nutritional resources for the chicken embryo, are principally digested and absorbed by the extra-embryonic tissues. These essential extra-embryonic tissues include the yolk sac, allantois, amnion, and chorion.

The yolk sac is the first extra-embryonic membrane, which advances from the embryo gut and gradually forms a membrane that encloses the yolk content during chicken embryogenesis. The yolk sac has three germ layers. The endoderm and mesoderm are important for nutrient absorption and hematopoiesis, respectively (Bauer et al. 2013; Sheng, 2010). The area and weight of the yolk sac reaches a peak around embryonic day (e) 15 and decreases towards hatch (Yadgary et al., 2013). Most of the nutrients in the yolk content gradually decline

during incubation due to uptake required for embryo development. However, the glucose amount in the yolk content unexpectedly increases at the end of embryogenesis (Yadgary et al., 2010). This surprising increase in glucose amount in the yolk is partially attributed to gluconeogenesis that occurs in yolk sac endodermal epithelial cells (Yadgary and Uni, 2012).

The chicken yolk sac is a multifunctional organ. Besides its function for nutrient uptake, yolk sac endodermal epithelial cells have also been found to function as hepatocytes in liver to produce critical plasma carrier proteins such as albumin, α-fetoprotein, lactotransferrin, hemopexin and some blood coagulation factors (Yadary et al., 2014). Additionally, at the end of incubation, the yolk sac could synthesize bile salts by using biliverdin, which is generated from heme catabolism in the yolk sac (Yadary et al., 2013; 2014). Although the yolk sac endodermal epithelial cells have not yet been shown to consist of different cell types, like the intestinal epithelial cells, which have enterocytes, goblet cells, Paneth cells, enteroendocrine cells, the yolk sac endothelium shares many similar biological functions with the small intestine. It has digestive, absorptive, and immune functions.

Absorptive function of the yolk sac

The yolk sac endoderm consists of a single layer of epithelial cells. The endodermal epithelial cells are connected with each other through cellular tight junctions. There are large lipid droplets distributed in the yolk sac endodermal epithelial cells and the different morphology of these lipid droplets divides the yolk sac into three areas: *area pellucida*, *area vasculosa*, and *area vitellina*. The *area vasculosa* of the yolk sac is the main absorptive site with a large number of blood vessels and greatest activity of lipid endocytosis and nutrient transport. During incubation, a number of digestive enzymes and nutrient transporters, which are normally

expressed by the intestine, are also differentially expressed in the yolk sac (YS) (Yadgary *et al.*, 2011, 2014; Speier *et al.*, 2012). Amino acids and short chain peptides were absorbed by the endodermal cells and transferred to the blood through specific nutrient transporters. In the chicken YS, mRNA expression of the di- and tri-peptide transporter PepT1 peaked at e15, followed by a gradual decease to day of hatch (doh). The YS also expressed peptidases and amino acid transporters (Yadgary *et al.*, 2011; Speier *et al.*, 2012). The mRNA abundance of aminopeptidase N showed a similar expression pattern to PepT1, with peak expression around e15 to e17 that decreased to doh. One of the amino acid transporters, neutral amino acid transporter B^oAT, showed increased mRNA expression in the YS from e11 to e19 and then declined. The anionic amino acid transporter EAAT3, which transports aspartate and glutamate, showed increased expression from e11 to doh (Yadgary *et al.*, 2011; Speier *et al.*, 2012). Other amino acid transporters and solute carriers (SLC) were also analyzed (Yadgary *et al.*, 2014). The important sugar transporter SGLT1 and the digestive enzyme sucrase-isomaltase (SI) were highly expressed at the end of the chicken embryonic stage (Yadgary et al., 2011). This supports the hypothesis that the chick needs increased energy from glucose for the hatching process.

Lipid metabolism in yolk sac epithelium

During chicken embryogenesis, the chick derives nutrients mostly from the yolk and albumen. In the egg yolk, the major nutrient component is lipoproteins, which are derived from liver in hens. The yolk sac endodermal epithelium is the main site for digestion and absorbs the yolk lipids for chicken embryo growth. These yolk sac epithelial cells in the *area vasculosa* assimilate the yolk derived, very low density lipoprotein or free yolk droplets mainly through receptor-mediated endocytic processes (Bauer et al., 2013). The highly expressed apolipoprotein

and lysosomal genes in the yolk sac endodermal epithelial cells revealed the potential function that yolk sac epithelial cells are capable of resynthesizing and secreting lipoprotein (Bauer et al, 2013; Yadgary et al, 2014). Another important pathway for yolk sac to metabolize yolk fat is to break down triglycerides into fatty acids and glycerol by lipase cleavage. The general fatty acid composition in yolk fat is about 0.9% (Deeming and Ferguson, 1991), but the amount of free fatty acid in yolk would change during incubation due to interconversion or lipid metabolism (Latour et al., 1998; Yadgary 2013). Besides the lipase that was detected in the yolk sac, bile salt was also found to be present at the last week of incubation in the chicken yolk sac. Both of these support the ability of the yolk sac to hydrolyze lipids into fatty acids (Yadgary 2013). However, the origin of bile salt in the yolk sac is still controversial. One hypothesis is that the bile salt is produced from the gall bladder through bile recycling, which passes through the intestine to the yolk content (Surai and Speake, 1998). However, Yadgary et al. (2013) suggested that the higher amount of bile salt in the yolk sac compared to the yolk content might indicate the ability of the yolk sac to synthesize bile salt. With evidence that the yolk sac is capable of secreting digestive enzymes, which are functionally secreted by the liver, the yolk sac might possess the same function as liver to form and secrete bile salts (Speier et al. 2012; Yadgary and Uni, 2012; Yadgary et al., 2014).

Glucose metabolism in the yolk sac epithelium

The glucose concentration in the newly fertilized egg is barely detectable yet still essential for development of the early chicken embryo (Jin et al., 2013). The chicken yolk sac is an important organ for generating blood cells and blood vessels. Jin et al. (2013) found that the low glucose concentration during early embryonic stage in chickens was critical to maintain yolk

sac angiogenesis and hematopoiesis. Additionally, they also demonstrated that the low glucose concentration decreased reactive oxygen species (ROS), which facilitated the growth of embryo cardiomyocytes and stimulated cardiogenesis (Jin et al., 2013). In the late stage of incubation, glucose and glycogen concentrations in the yolk sac increased from e11 to e19 (Yadgary and Uni, 2012). At the same time at e19, both Yadgary et al. (2011) and Speier et al. (2012) found elevated mRNA expression of the glucose transporter SGLT1 and slightly increased expression of sucrase isomaltase (SI) in the chicken yolk sac. The high expression level of key enzymes in the yolk sac that are involved in gluconeogenesis indicates that yolk sac epithelial cells could generate glucose that is stored as glycogen in the cell, which is similar as the synthesis of glycogen by hepatocytes through the gluconeogenic pathway (Yadgary and Uni, 2012). At the end of the embryonic stage, when the yolk sac degrades, one of the pivotal changes of the yolk sac is the apoptotic fracture of yolk sac epithelial cells, which releases glycogen into the yolk content (Yadgary et al. 2014). The glucose amount later increases in the yolk content through glycogenolysis, which provides energy for the chicken embryo to hatch (Yadgary and Uni, 2012).

Protein and oligopeptide metabolism in yolk sac epithelium

The yolk sac endoderm plays a critical role in using yolk proteins to synthesize serum proteins and construct the yolk sac vascular system (Nakazawa et al. 2011). In fact, the consumption of yolk proteins by the yolk sac is hard to measure because the proteins are continuously exchanged between the albumen and the yolk during embryogenesis (Yadgary et al. 2010). The yolk protein is mainly used to produce very low density lipoproteins, which are in the form of soluble lipovitellin or phosvitin. Thus, one of the potential ways for the yolk sac to

absorb proteins is through the uptake of lipoproteins. In addition, Speier et al. (2012) have found that the chicken yolk sac expressed aminopeptidase N (APN), which hydrolyzes neutral amino acids from the N terminus of proteins, showing the ability of the yolk sac to digest proteins. The developmental expression of neutral amino acid transporter B^oAT, cationic amino acid transporter CAT1, and oligopeptide transporter PepT1 in the yolk sac further demonstrated that the yolk sac was capable of metabolizing yolk proteins during chicken embryogenesis (Yadgary et al., 2011; Speier et al., 2012).

Immune function of chicken yolk sac

The yolk sac epithelium is able to secrete avian beta defensins (AvBD), which are important host defense antimicrobial peptides for boosting chicken innate immunity to protect against exogenous pathogens (Yadgary et al., 2014). Zhao et al. (2001) found that expression of AvBD3 mRNA in the yolk sac was too low to detect by using end-point PCR. However, the detection of AvBD10 mRNA in the yolk content might indicate the synthesis of antimicrobial peptides either from the yolk sac or from maternal nutrient-deposition (Xiao et al., 2004).

Avian beta defensins

Different from mammals, which have both alpha and beta defensins, avian species have only beta defensins. Avian beta defensins are small cationic and amphipathic peptides enriched with disulfide bridges and beta sheets in the protein tertiary structure (Ganz, 2004). The wide antimicrobial spectrum of avian beta defensins allows them to serve as an important component in the avian innate immune system (Sugiarto and Yu, 2004). In chickens, 14 AvBD genes have been detected in the genome and are named from AvBD1 to 14. Chicken beta defensins were

first isolated from chicken heterophils, which play a similar function as neutrophils in humans (Evan et al., 1995). Mass spectrometry analysis of small proteins (less than 20 kDa) in chicken heterophils revealed that AvBD2 was a peptide enriched in heterophils and would serve as a biomarker for chicken heterophils (Kannan et al., 2009). Besides leukocytes, Cuperus et al. (2016) performed immunohistochemistry and found that AvBD9 protein was expressed by intestinal epithelial cells, which are presumed to be enteroendocrine cells. Additionally, AvBD mRNA exhibited a tissue-specific expression pattern in chickens. AvBD1, 2, 6, and 7 mRNA were found to be highly expressed in chicken bone marrow (Lynn et al., 2004; Lee et al., 2016). The expression of AvBD3 mRNA was strongly expressed in bursa and tongue (Zhao et al., 2001; Lynn et al., 2004). Since the critical immune organs responsible for adaptive immunity are not completely developed during the embryonic and early posthatch periods, the innate immune system of beta defensins plays an important role for chicken early immunity. The expression of AvBD mRNA in the chicken embryo has been investigated during the early embryonic stage (Meade et al., 2009). Developmentally, the abundance of AvBD2, 6, and 7 mRNA increased from e3 to e6, decreased from e6 to e9, and increased from e9 to e12. During the early posthatch stage, the development of the jejunum was found to be associated with increased expression of AvBD2 and 7 mRNA (Schokker et al., 2009).

The transcriptional expression of AvBD in the gastrointestinal tract could be induced by infection with pathogenic microorganisms. Chickens infected with *Salmonella* Enteritidis or Typhimurium showed higher expression of AvBD mRNA compared with control birds (Akbari et al., 2008; Milona et al., 2007). During necrotic enteritis, chickens that were infected with both *Eimeria* and *Clostridium perfringens* increased expression of AvBD8, 11 and 13 mRNA in the jejunum (Hong et al., 2012).

Hematopoietic function of chicken yolk sac

In the chicken, the yolk sac mesoderm is the major expansion and differentiation site for hematopoiesis during early chicken embryogenesis and cooperates to produce blood cells with the bone marrow during the late embryonic and early posthatch stages (Sheng, 2010). The hematopoietic process in chicks is different from other animals in that the fetal liver is the major producer of blood cells with spleen and bone marrow active during the second half of gestation (Godin and Cumano, 2005). Conversely, chicken liver does not contribute to the generation of erythrocytes.

During chicken embryogenesis, the earliest blood islands in the form of aggregation of blood and vascular precursors in the yolk sac appear between e0.5 to e1. Later, the yolk sac derived primitive erythrocytes begin to differentiate into vascular endothelial cells and some primitive blood from e1 to e1.5, followed by establishment of the blood circulation at e2 (Sheng, 2010). The shift of the hemoglobin composition (Alev et al., 2008) and erythrocyte morphology (Romanoff, 1960) at e5 proves the gradual disappearance of primitive erythropoiesis and the presence of definitive erythropoiesis during the embryonic stage. Primitive and definitive erythropoiesis are two erythropoietic processes that serve at different stages, but both of them have to undergo the same maturing stages. The subsequent definitive erythropoiesis is still observed up to e19 (Niimi et al. 2008). Two kinds of cell types on the yolk sac were assumed as the main sources for definitive erythrocytes, the vascular endothelial cells and primitive erythrocytes. Some molecular marker studies supported the endothelial-derived hypothesis. Additionally, some recent research found that endothelial cells were able to generate hematopoietic cells in the dorsal aorta, which also indicates the erythropoietic potential of the vascular endothelium (Bertrand et al., 2010). Nagai and Sheng (2008) found the time of initiation

of definitive erythropoiesis corresponded to the second phase of yolk sac vascular formation, suggesting that the yolk sac endothelial cell might be able to generate erythropoietic cells. Another hypothesis was that the primitive erythrocytes might dedifferentiate into definitive erythrocytes. The functional transition from primitive erythrocyte to definitive erythrocyte at e5 may provide evidence to support the second hypothesis.

Function and structure of small intestinal epithelium

The small intestinal mucosal epithelium is derived from chicken embryonic endoderm and is critical for digestion and absorption of nutrients from the diet. Physiologically, the chicken small intestine is divided into duodenum, jejunum, and ileum. The duodenum forms a "U" like structure that has the pancreas attached to its serosal layer. The end of the duodenum is the site where pancreatic ducts and bile ducts converge. The pancreas and gall bladder are able to secrete digestive enzymes and bile salts through the ducts into the small intestine to facilitate the break-up and assimilation of dietary nutrients. The jejunum sits in the middle of the small intestine and serves as a transition from the duodenum to ileum. The site that the yolk sac connects to the small intestine through the yolk stalk is Meckel's diverticulum, which is also the location demarcating the jejunum and ileum (Yamauchi, 2002). One of the most prominent features that differentiates the small intestine from the colon is the large number of villus structures that protrude into the lumen of the small intestine compared to the colon. In mammals, the intestinal villus is lined with epithelial cells, which consist of different cell types including enterocytes, goblet cells, Tuft cells, and enteroendocrine cells (van der Flier and Clever, 2009). The intestinal stem cells and Paneth cells are located in the intestinal crypts, which are at the base of the intestinal villus. The different cell types can all be identified by using specific marker genes

(Barker, 2014). There are different molecular signaling pathways controlling the differentiation and stemness of the intestinal stem cells, with Wnt/β-catenin signaling and Notch signaling being the most important. The mature epithelial cells migrate along the intestinal villus and are shed into the intestinal lumen when they reach the tip of the villus (Sato and Clever; 2013).

The intestinal crypt forms a niche

The intestinal crypt, which was first identified by Jonathan Lieberkühn through histological analysis, is the site of cells with proliferating ability to renew the intestinal epithelium. These cells have been well characterized and defined as intestinal stem cells that are controlled by complicated cell signaling pathways to maintain stemness and differentiation into absorptive or secretory cell lineages. In the mammalian model, the intestinal crypt niche is marked by Paneth cells interspersed with stem cells (Figure 2.1). This special pattern makes Paneth cells indispensable for intestinal stem cell growth and differentiation, including providing Notch ligands, Wnt proteins and growth factors (Sato et al., 2011; Sato and Clever; 2013). Additionally, Paneth cells are important for maintaining homeostasis of the intestinal crypt niche by secreting antimicrobial peptides and lysozymes (Peeters and Vantrappen; 1975; Tokiyoshi et al., 2000; Charles and Nita 2011). Lgr5 and Olfm4 are the two most popular intestinal stem cell markers that are used to identify the ontogeny and distribution of intestinal stem cells in mammals (van der Flier and Clevers, 2009).

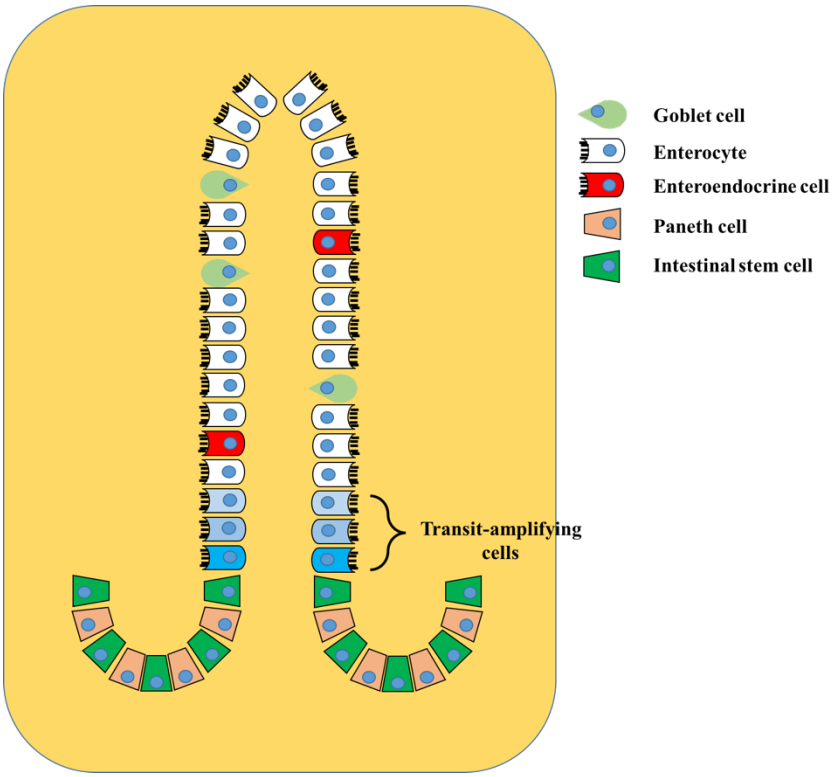

Figure 2.1 Cell types along intestinal crypt-villus axis in mammals (figure drawn by H. Zhang)

Intestinal stem cell marker Lgr5

Leucine-rich repeat-containing G protein coupled receptor 5 (Lgr5) is a widely used biomarker in mammals and rodents for labelling intestinal stem cells. Lgr5 is able to bind the extracellular R-spondins, which are mostly secreted by intestinal stroma and stimulate the Wnt/β-catenin Tcf downstream signaling pathway (de Lau et al., 2011). Lgr5 knock-out homozygous mice were not able to survive after birth due to abnormality in the gastrointestinal tract and tongue (Morita et al., 2004). The expression of Lgr5 is ubiquitous throughout the body around birth, suggesting the important role of Lgr5 in embryonic and early neonatal development (Kinzel et al., 2014). Later, Lgr5 expression is gradually restricted to a few cells in the intestine, stomach, hair follicle, mammary gland, eyes, and brain (Jaks et al., 2008; Barker et al., 2010; de

Visser et al., 2012). In vitro culturing of $Lgr5^+$ intestinal stem cells has been successfully used to establish an $Lgr5^+$ stem cell derived organoid that was capable of growing and differentiating into all other cell types (Sato et al., 2009). Recently, Lgr5 continues to be a hot topic because it is also able to mark intestinal cancer stem cells, which reside in intestinal adenomas and share a very similar structure to the intestinal crypts that are interspersed with Paneth cells (Schepers et al., 2012). Additionally, the fine structure of the intestinal stem cell population was well characterized, including the highly proliferating $Lgr5^+$ stem cells to maintain epithelium regeneration, and $Bmi1^+$ (B cell-specific moloney murine leukemia virus integration site 1) quiescent intestinal stem cells to recover from injury to intestinal stem cells (Yan et al., 2012).

Intestinal stem cell marker Olfm4

Olfactomedin 4 (Olfm4) is a secreted polymeric glycoprotein, which is a robust biomarker of intestinal stem cells (van der Flier and Clevers, 2009). It was first isolated from human hematopoietic myeloid cells and the mRNA expression pattern displayed a tissue-specific distribution. Olfm4 is also a widely used intestinal stem cell marker that is superior to Lgr5 due to its high expression of both mRNA and protein in the small intestinal crypts (Schuijers et al., 2014). Olfm4 has been found to be expressed in the intestinal crypt in human colon, but not in the murine colon (van der Flier and Clevers, 2009). In humans, expression of Olfm4 mRNA was also detected in the prostrate, bone marrow, and neutrophils (Zhang et al., 2002; Clemmensen et al., 2012). The functions of Olfm4 are largely focused on its effects on tumorigenesis, especially in gastrointestinal carcinogenesis. The downregulation of Olfm4 mRNA by using RNA interference in tumor cells in vitro significantly decreased the generation of gastric carcinomas, while the knock-out of Olfm4 did not interrupt tumorigenesis (Liu et al., 2012). Another

prominent finding is that the plasma level of Olfm4 protein in patients who have gastrointestinal cancer was elevated, which indicated that Olfm4 was promising as an important biomarker for diagnosing gastrointestinal cancer (Clemmensen et al., 2015). Additionally, Olfm4 is involved in the mucosal defense in the gastrointestinal tract. It serves a similar role as mucin to protect the intestinal epithelial cells (Gersemann et al., 2012). The biochemical functions such as binding to defensins also provide evidence that Olfm4, like mucin, plays important roles in the intestinal defense reaction (Gersemann et al., 2012).

Paneth Cells

Paneth cells were first discovered in 1888 in the intestinal crypt and are characterized by the trapezoidal shape of the cell, which has many cytoplasmic granules located at the base of the cell. The Paneth cell is identified through histological analysis including phloxine-tartrazine and eosin staining because it contains acidophilic granules, which are concentrated with cationic charged proteins or peptides. Paneth cells appear in both the small intestine and colon in humans beginning with the second trimester of pregnancy. In mice and rats, Paneth cells do not show up until the intestinal crypts are completely formed after birth. The presence of Paneth cells in mammals and other lower vertebrates is dependent on the species (Porter et al., 2002). Some animals were found to not have Paneth cells in the crypts, instead having other intestinal cell types along the villus functioning like Paneth cells (Reilly et al., 1994). In chickens, immunohistochemical localization of lysozyme, which is a widely used biomarker for labelling Paneth cells, showed that lysozyme was distributed along the small intestinal villus of 17 day old chickens, indicating the absence of Paneth cells in the intestinal crypts (Nile et al., 2004). However, Paneth cells were found to be present in the small intestine of 6-month-old chickens

when treated with histological staining and *in situ* hybridization of lysozyme (Wang et al., 2016). In other avian species such as ostrich, Paneth cells were found to be absent (Porter et al. 2002).

Paneth cell interacts with the intestinal stem cell

In mammals, Paneth cells are differentiated from stem cells and intermingled with stem cells in the intestinal crypt. The intimate spatial relationship reflects the indispensable role of Paneth cells for the development and differentiation of intestinal stem cells. Single cell culture of intestinal stem cells can generate intestinal organoids or "mini guts" in vitro by providing the secreted growth factors from Paneth cells (Rodríguez-Colman et al. 2017). Molecular analysis of $CD24^+$ Paneth cells suggested that Paneth cells participate in many critical cellular signaling pathways that maintain intestinal stem cell growth and differentiation by secreting factors such as EGF (Epidermal Growth Factor), TGF-α (Transforming Growth Factor-α), Notch ligand Dll4, and Wnt signaling binding protein Wnt-3 (Sato et al., 2011). Besides providing crucial cellular signaling factors for the intestinal stem cells, Paneth cells are also capable of secreting defensins, lysozyme c, and other antimicrobial peptides to maintain homeostasis of the intestinal microbiome (Peeters and Vantrappen et al., 1975; Porter; 2002; Salzman et al., 2010). Additionally, cellular analysis revealed an abundant endoplasmic reticulum and active Golgi network, which is necessary for an intensive intracellular protein synthesis and high extracellular secretory activity of Paneth cells (Bevins et al., 2011).

Intestinal absorptive enterocytes

The enterocyte represents the largest intestinal epithelial cell population (>80%) and mainly participates in nutrient digestion and absorption (Cheng and Leblond; 1974). The

polarized enterocytes along the intestinal villi connect to each other through tight junctions and form a physiological barrier to recognize and exclude exotic antigens from digestible nutrients. The surface of the enterocyte apical membrane is expanded as microvilli, which largely increases the surface area of the enterocyte (Phillips et al., 1979). The capability of enterocytes to clean antigens or macronutrients that are engulfed by cellular endocytosis was consistent with the high protein sorting and lysosomal degradation occurring in the cell (Mostov et al., 1992). Additionally, the mucus coat of the enterocytes significantly increases surface viscosity, thus effectively decreasing the precipitation rate of macromolecules, like antigens and nutrients (Strocchi and Levitt, 1991). In chickens, mature enterocytes with tight junctions and columnar morphology are present at day of hatch. The embryonic enterocyte appears in the form of a goblet cell, which is distributed at the base of the epithelium (Karcher and Applegate, 2008).

Regulation of enterocyte differentiation

The enterocytes are derived from absorptive progenitors, which differentiate from intestinal stem cells and play a major function for absorbing nutrients. The differentiation of intestinal stem cells is either towards the intestinal absorptive cell lineage including enterocytes or intestinal secretory cell lineage including Paneth cells, goblet cells, and enteroendocrine cells. Both the absorptive and secretory cell lineages are primarily mediated by Notch and Wnt signaling pathways. Alkaline phosphatase intestinal gene (Alpi) is a common biomarker for identifying the lineage tracing of the enterocytes. Normally, Alpi+ enterocytes develop from the intestinal crypt, move up along the intestinal villus, and finally are shed into the intestinal lumen from the tip of the villus (Lalles et al., 2010). However, an elegant study was conducted recently to support the plasticity of the enterocyte progenitors, which were able to migrate down to the

intestinal crypts to compensate for the loss of intestinal stem cells induced by injury (Tetteh et al., 2016).

Nutrient transport systems in enterocytes

In addition to endocytosis of macronutrients and pathogens, the uptake of micronutrients like oligopeptides, amino acids, sugars, and minerals by enterocytes is mainly mediated through nutrient transporters. Nutrient uptake by the enterocytes is mediated by the transporters located on the brush border membrane and basolateral membrane. Most of the micronutrients are transported by consuming energy or utilizing the driving force produced from an electrochemical gradient. The substrates absorbed by the brush border membrane transporters are available to be metabolized into smaller molecules, resynthesized into other compounds in the enterocyte or transported into the blood through the basolateral membrane (Gilbert et al., 2008; Montagne et al., 2004). The transporter-mediated absorption of nutrients by enterocytes might also be controlled by other extra- or intra-cellular factors. For example, the absorption of iron was mediated by the divalent metal-ion transporter 1 on the brush border membrane of the enterocyte and responded to the change of intracellular iron and oxygen content (Fuqua et al., 2012). The transport of fatty acids by enterocytes was affected by the microbiota in the intestinal mucosal layer (Semova et al., 2012).

The sodium-dependent glucose cotransporter (SGLT1) is important for glucose uptake in chickens. Gene expression of SGLT1 increased from e19 to e21 in the embryonic small intestine (Speier et al., 2012). The jejunum had the greatest abundance of SGLT1 mRNA compared to duodenum and ileum in the small intestine of broiler chickens posthatch (Gilbert et al., 2007).

SGLT1 in glucose uptake

The sodium-dependent glucose cotransporter is encoded by the SLC5 gene family. The SGLT protein serves as a transporter to mediate the Na^+ coupled transport of glucose down an electrochemical gradient. In humans, the SLC5 gene family contains six family members, and SGLT1 (SLC5A1) has been found to be the most important for mediating glucose uptake in the intestine (Wright, 2013). Polysaccharides are broken down into oligosaccharides by the enzymatic cleavage with α-amylases. These oligosaccharides are further digested into monosaccharides including glucose and galactose, which are absorbed mainly in the small intestine by SGLT1, which is predominantly expressed and located at the brush border membrane of enterocytes (Garriga et al., 1999; Barfull et al., 2002). The human SGLT1 protein has 664 amino acids, which anchor in the lipid bilayer of the cell membrane and form 14 putative transmembrane domains (Wright et al., 2011). The driving force for SGLT1 to transport glucose or galactose from the intestinal lumen into enterocytes is generated by cotransporting sodium into the enterocyte down the sodium gradient, which is maintained by the sodium-potassium ATPase on the basolateral membrane (Drozdowski and Thomson, 2006). In addition to SGLT1, fructose is transported by GLUT5 which is a Na^+ independent fructose transporter located on the brush border membrane of the enterocyte. At the basolateral membrane of the enterocyte, another Na^+ independent monosaccharide transporter, GLUT2 is responsible for transporting many monosaccharides into the blood stream and is also capable of carrying nutrients from the blood stream to the enterocytes (Shirazi-Beechey, 1995).

Regulation of SGLT1 expression

In humans and rats, the expression of SGLT1 shows a very similar pattern of highest expression level in duodenum and jejunum, and lowest expression in the distal small intestine (ileum) (Chen et al., 2010; Balen et al., 2008). However, the expression of SGLT1 did not show any differences from duodenum to ileum in dogs (Batchelor et al., 2011). The high glucose concentration in contact with the enterocytes could induce increased expression of SGLT1. Besides glucose, the carbohydrate concentration also affects the expression of SGLT1 in the small intestine of animals. Induced expression of SGLT1 in the pig ileum resulted from feeding the animal with slow digestible starch (Woodward et al., 2012). However, in rats, SGLT1 expression was only elevated in jejunum when the animal was fed with high-fructose or -sucrose diets (Kishi et al., 1999).

Peptide transporter, PepT1

The proton-coupled oligopeptide transporter 1(PepT1), which is encoded by the SLC15A1 gene, serves an important role in absorbing amino acids as short peptides in the digestive tract. PepT1 is highly specific for transporting di- and tri-peptides or peptide-like substrates in the small intestine (Spanier, 2014). PepT1 is a low affinity, high capacity transporter compared to the high affinity, low capacity transporter PepT2. The high efficiency of PepT1-mediated transport makes it an optimal transporter to absorb some peptide-like pharmaceutical drugs, such as β-lactam antibiotics, the anticancer drug bestatin, and the antiviral prodrug valacyclovir (Matthias et al., 2008). Chicken PepT1 cDNA was first characterized by Chen et al. (2002) in *Xenopus* oocytes and Chinese hamster ovary cells. Chicken PepT1 consists of 714 amino acids. The expression of PepT1 in mammals and rodents was predominantly

distributed in the small intestine compared with other tissues. However, PepT1 was also found to be expressed in the epithelium of extrahepatic biliary duct, pancreas, and the reproductive organs (Knutter et al., 2002; Bockman et al., 1997; Lu and Klaassen, 2006). Further comparison with different parts in the small intestine in chickens, showed that the expression profile of PepT1 mRNA was greatest in the duodenum (Zwarycz and Wong, 2013).

Regulation of PepT1 expression

In different physiological and pathological conditions, the expression of PepT1 could be distinctly controlled. Upregulated PepT1 expression was observed when rats were fed with a high protein diet or rats were starved (Naruhashi et al., 2002; Adibi, 2003). Snorre et al. (2010) also found higher expression of PepT1 mRNA in fish small intestine when fish were fed with free amino acids or ultrafiltered hydrolysate peptides. In addition, the expression of PepT1 showed a differential response to dietary changes in different parts of the fish small intestine. In chicken and rodents, PepT1 expression still exhibited upregulation even though the animals were supplied with a low protein or amino acid diet (Ogihara et al., 1999; Ihara et al., 2000; Madsen and Wong, 2011; Ma et al., 2012). This elevation of PepT1 expression during protein deficiency or fasting has been found to be associated with the effect of peroxisome proliferator-activated receptor α (PPARα) in chickens (Madsen and Wong, 2011). Shimakura et al. (2006), however, found that PepT1 expression was not changed when PPARα knock-out mice were fasted.

Intestinal Goblet cells

One of the most important intestinal secretory cell types along the crypt-villus axis to maintain homeostasis is the goblet cell. The major function of intestinal goblet cells is to secrete

mucins and other products, which together form the mucus layer, an innate immune defense barrier, to protect the intestinal epithelia. The name of the goblet cell is characterized by its special cell morphology, which has distended theca accumulating with the highly glycosylated granules close to the basal membrane (Specian and Oliver, 1991). The intestinal goblet cells are derived from intestinal stem cells, and this process has been shown to be mainly controlled by Notch signaling (van der Flier et al., 2009). The suppression of Notch signaling leads to the propagation of secretory cells like goblet cells, Paneth cells, and enteroendocrine cells that differentiate from intestinal stem cells (Stanger et al., 2005). The terminal differentiation of intestinal goblet cells is regulated by different transcriptional factors such as Math1, Klf4 and Elf3 (van der Flier et al., 2009). The proportions of goblet cells along the intestinal villus from duodenum to ileum generally increase due to the presence of microbes close to the distal intestine. In chickens, the potential goblet cells in the intestine were found to be present early at embryonic day 17, but only contained acidic granules. The percentage of mature intestinal goblet cells in the chicken are gradually increased from duodenum to ileum in percentage, and the morphology of the goblet cells during the posthatch stage are well-formed and enclosed with both acidic and neutral mucin (Uni et al., 2003). Additionally, recent findings indicated that melatonin might be a positive factor to inhibit Notch signaling, which can further increase the goblet cell population in the chicken (Li et al., 2017).

Intestinal goblet cells secrete Muc2

Muc2 mRNA is the most abundant mRNA expressed by goblet cells in both the small intestine and colon (Andrianifahanana et al., 2006). The mucin genes are in a large gene family and categorized into two different branches, secretory mucins and membrane-binding mucins.

The Muc2 protein is a serine and threonine enriched glycoprotein, which forms a trimer with extensive O-linked glycosylation and is secreted out of the cell in the form of large and heavy polymers (Lidell et al. 2003). The alteration of Muc2 mRNA expression significantly changes the morphology of goblet cells when other components synthesized by goblet cells are normally presented (Velcich et al., 2002). The synthesis of Muc2 was found to be regulated mainly through affecting three transcriptional factors including Nuclear factor (NF)-κB, CREB/ATF1, and SAM pointed domain-containing Ets (SPDEF) (Li et al., 1998; Hokari et al., 2005; Gregorieff et al., 2009).

Enteroendocrine cell

The gastrointestinal tract has the largest endocrine system in regulating gut-brain and brain-gut neuroendocrine bidirectional interactions. Enteroendocrine cells, even though they are only a small cell population (about 1%) along the intestinal crypt-villus axis, play a very important role in regulating gastrointestinal homeostasis (Rehfeld et al., 2004). Enteroendocrine cells, which are derived from the intestinal secretory cell lineage, contribute greatly to hormone modulation and neuroendocrine regulation. Enteroendocrine cells are derived from the intestinal crypt and gradually migrate up along the intestinal villus. The differentiation of enteroendocrine cells is mainly mediated by the extracellular signaling proteins like notch and the helix-loop-helix transcriptional factor family (Artavanis-Tsakonas et al., 1999; Li et al., 2011; Beehler-Evans and Micchelli 2015; Wolnerhanssen et al., 2017; Sakar, 2014). The accumulated protein or peptides in the enteroendocrine cells are released into the blood stream from the basolateral membrane by sensing changes in the intestinal lumen. The secreted hormones and peptides could stimulate cell activities close to the enteroendocrine cell through a paracrine process, or trigger

the activation or inhibition of cells at a distance through trafficking in the blood circulation (Psichas, 2015). The enterendocrine cells were also able to transmit the nerve impulse since they have been confirmed to have synaptic structures and glia cells attached (Bohorquez et al., 2014). One of the major components that enteroendocrine cells secrete is cholecystokinin (CCK), which often serves as a marker for researching enteroendocrine cells. However, there are different types of enteroendocrine cells secreting different bioproducts. CCK-secreting enteroendocrine cells were also producing gastric inhibitory peptide (GIP), secretin, somatostatin, and glucagon-like peptide-1 (GLP1). GLP1 is another popular biomarker for labelling enteroendocrine cells (Egerod et al, 2011).

Summary

The chicken embryo develops for approximately 21 days. In the early stage of chicken embryogenesis, the yolk sac produces blood cells through primitive and definitive hematopoietic processes. During the last two weeks, the yolk sac functions mainly for nutrient digestion and absorption. Finally, apoptosis of both yolk sac endoderm and mesoderm occurs close to the end of hatch, but the small intestine is developing during this time and eventually will take the place of the yolk sac to metabolize nutrients for embryo development.

The chicken yolk sac absorbs nutrients mostly from the egg yolk which consists of 33% lipid, 15% protein, and 1% carbohydrate. The egg yolk is a unique fat resource that contains very low density lipoproteins synthesized by the maternal liver. The lipid content in the yolk generally decreases during chicken embryogenesis due to the utilization of this lipid through beta oxidation by the embryo. However, the amount of protein in the yolk content fluctuates because of the

transfer of albumen into the yolk. Gluconeogenic and glycogenolytic processes in the yolk sac increase the glucose content in the yolk at the end of hatch

Endodermal epithelial cells of the chicken yolk sac express nutrient transporters mainly during the last 10 days of incubation. The expression of PepT1 mRNA was the highest at e13 to e15 when the yolk sac reaches its maximal absorptive area. Increasing glucose amount in the yolk at the end of hatch might boost the expression of SGLT1 mRNA in the yolk sac epithelial cells. There are also amino acid, mineral, and other sugar transporters expressed by yolk sac endodermal epithelial cells indicating high nutrient uptake in the yolk sac epithelium.

The mesoderm of chicken yolk sac is the site for generating blood cells. In the yolk sac, definitive erythrocytes are present even during the late stage of chicken embryogenesis, suggesting that the yolk sac has continuous hematopoietic ability. However, the shrinkage of blood vessels and cells was observed at the end of the embryonic stage.

The small intestinal epithelium is the major site for digestion and absorption of nutrients during the late embryonic and post-hatch stages. Nutrient uptake is primarily mediated by enterocytes, which is the largest cell population along the intestinal villus. Except for absorptive cells on the intestinal villus, there are also different kinds of secretory cells such as enteroendocrine cells which produce digestive enzymes, and hormones, and goblet cells which produce mucins. Paneth cells are also one cell type in the intestinal secretory cell lineage, but they are predominantly distributed in the intestinal crypts in mammals and rodents. Both these absorptive and secretory cells on the intestinal crypt-villus axis originate from stem cells in the intestinal crypts. These processes are regulated by different cell signaling pathways.

Lgr5 has been used as a marker for identifying intestinal stem cells. The expression of Lgr5 was also found in other tissues and is represented as a wide stem cell marker. Recent

studies also found that $Lgr5^+$ adenomatous cells were likely cancer stem cells that were capable of infinitely generating intestinal tumor cells. Olfm4 is another intestinal stem cell marker, which is expressed much higher than Lgr5 in the intestinal crypt. Thus, Olfm4 is a more robust gene than Lgr5 as an intestinal stem cell marker.

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Chapter III

Hypotheses and Objectives

In mammals, there are different cell types along the intestinal villus, which are responsible for the multiple functions of the intestinal epithelium. However, the cell populations in chicken intestinal epithelia have not yet been identified. The chicken yolk sac shares many similarities to the small intestine. The yolk sac has been recognized as a multifunctional organ, which is able to absorb nutrients, generate blood cells and secrete immune factors. But, which cell in the yolk sac is responsible for each function is still unknown. To address these questions, the hypotheses and objectives of this study are described below.

Hypotheses:

1. The organization and ontogeny of cells in the chicken small intestine is similar to mammals, with the enterocytes located along the villi and stem cells located in the crypts.

2. The chicken yolk sac contains many cell types, which are able to absorb nutrients, produce blood cells, and secrete innate immune factors.

Objectives:

1. Identification of cells expressing stem cell markers (Olfm4, Lgr5), nutrient transporters (PepT1, SGLT1) and host defense peptides (AvBD10) mRNA in the chicken small intestine from the late embryonic to early posthatch stages.

2. Identification of cells expressing stem cell markers (Lgr5), nutrient transporters (PepT1, SGLT1) and host defense peptides (AvBD1, 2, 7, and 10) mRNA in the chicken yolk sac at different times of incubation.

Chapter IV

Spatial transcriptional profile of PepT1 mRNA in the yolk sac and small intestine in broilers

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ABSTRACT

The yolk sac and small intestine are two important organs responsible for the digestion and absorption of nutrients in chickens during the embryonic and posthatch periods, respectively. The peptide transporter PepT1 is expressed in both the yolk sac and small intestine and plays an important role in the transport of amino acids as short peptides. The objective of this study was to profile the spatial transcriptional patterns of PepT1 mRNA in the yolk sac and small intestine from embryonic and posthatch broilers. The distribution of PepT1 mRNA was investigated by *in situ* hybridization at embryonic day (e) 11, 13, 15, 17, 19 and day of hatch (DOH) in the yolk sac and at e19, DOH, D1, D4 and D7 in the small intestine. PepT1 mRNA was expressed in the endodermal cells of the yolk sac. Expression of PepT1 mRNA was barely detectable at e11, increased from e11 to e13, e15 and e17, and then gradually decreased from e19 to DOH. In the small intestine, there was a rapid increase in expression of PepT1 mRNA in the enterocytes from e19 to DOH, with expression relatively constant from D1 to D7. In addition, there was a differential increase in the lengths of the villi in the different parts of the small intestine from D1 through D7, which may partially explain the temporal increase in PepT1 detected by qPCR. The villi in the duodenum showed the earliest increase in villus length and ultimately resulted in the longest villi at D7. These results demonstrated that there are temporal changes in PepT1 mRNA

expression in the yolk sac and the small intestine, which correspond with their expected role in nutrient uptake during the embryonic and posthatch periods.

Key words: PepT1, yolk sac, intestine, *in situ* hybridization, chicken

INTRODUCTION

The chick derives its nutrients from the yolk during embryogenesis and at hatch transitions to acquire nutrients from feed in the intestine. Both the yolk sac (YS), which surrounds the yolk and the intestine contain absorptive cells that are important for the uptake of nutrients, such as amino acids, peptides, fats and monosaccharides (Moran, 2007). Efficient uptake of nutrients is important to optimize growth and development of the embryonic and post hatch chick.

In the first week of chick embryonic development, endodermal epithelial cells (EECs) advance from the gut of the embryo and gradually spread over the surface of the yolk forming the YS; thus, the intestine and YS form a contiguous membrane (Mobbs and McMillan, 1979). These ECCs, which are directly in contact with the yolk contents, are responsible for absorbing nutrients to be transported to the blood circulation for the chick embryo (Mobbs and McMillan, 1981; Noble and Cocchi, 1990). The YS is not just a membrane that surrounds the yolk but is a multifunctional organ that provides the function of organs that have not yet developed. For example, the YS serves as the bone marrow in synthesis of blood cells, the intestine in digestion and transport of nutrients and lipids, the liver in production of plasma carrier proteins and carbohydrates, and the immune system in production of antimicrobial peptides (Yadgary and Uni, 2012; Yadgary et al., 2014). A number of digestive enzymes and nutrient transporters

normally associated with the intestine are differentially expressed in the YS (Yadgary et al., 2011, 2014; Speier et al., 2012).

During the last days of embryogenesis, the chick embryo swallows some of the amniotic fluid, which provides nutrients that are digested and absorbed by the developing intestine (Uni and Ferket, 2004). During this time, there is upregulation of digestive enzymes and nutrient transporter mRNA in the embryonic small intestine (Uni et al., 2004; Gilbert et al., 2007; Li et al., 2008; Speier et al., 2012; Zwarycz and Wong, 2013). After hatch, the small intestine continues to increase in size and develop its characteristic morphological structure (Sklan and Noy, 2000; Noy et al., 2001).

The uptake of peptides is mediated by PepT1, which is a proton-coupled oligopeptide transporter. PepT1 is a low affinity, high capacity transporter of di- and tri peptides and plays an important role in the uptake of amino acids (Adibi, 2003; Gilbert et al., 2008; Spanier, 2014; Daniel and Zietek, 2015). In chickens, PepT1 mRNA showed the greatest expression in the small intestine compared to other tissues (Zwarycz and Wong, 2013). Within the small intestine, PepT1 mRNA was expressed greater in the duodenum compared to the jejunum and ileum (Gilbert et al., 2007). PepT1 showed temporal changes in gene expression with an increase from late embryogenesis (e19) to posthatch (Gilbert et al., 2007; Li et al., 2008; Speier et al., 2012, Yadgary et al., 2012). PepT1 also showed temporal-specific expression in the YS with a rapid rise from e11 to e15 and then a gradual decline from e15 to DOH (Speier et al., 2012; Yadgary et al., 2011).

To date all of these studies profiling PepT1 mRNA expression in the YS and intestine of chickens has used qPCR. The localization of PepT1 mRNA expression in intestinal cells has not been determined in chicken. Thus, the objectives of this study were to determine temporal and spatial changes in PepT1 mRNA expression by *in situ* hybridization in the YS from e11 to day of hatch and in the intestine from e19 to D7 post hatch.

MATERIALS AND METHODS

Animals and Tissue Collection and Processing

Commercial broiler (Cobb 500) eggs were transferred from the hatchery (Longenecker's Hatchery Inc., Elizabethtown, PA) to Virginia Tech and placed into incubators (OVA-Easy Advanced, Brinsea, Titusville, FL). All eggs were incubated at 38°C and 36% relative humidity. The eggs were automatically turned once every 2 hours. Unfertile eggs were removed by candling at embryonic day 10 (e10). Embryonic and post-hatch chicks were killed by cervical dislocation. All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech. The area vasculosa of the YS was separated and cut into 2-3 cm^2 pieces after completely washing away the yolk contents in sterile 1X PBS on e11, e13, e15, e17, e19, and day of hatch (DOH). The small intestine was collected on e19, DOH, and D1, D4, and D7 post-hatch and separated into duodenum, jejunum, and ileum. Each part of the small intestine was rinsed with 1X PBS and completely removed of mesentery. Six samples per time point were collected for both YS and small intestine.

After separation and cleaning of the samples, the YS and the small intestine were fixed in phosphate buffered 4% paraformaldehyde for 24 hours. The tissues were stored in 70% ethanol

at 4°C for 24-36 hours after fixation and then sent to Histo-Scientific Research Labs Inc. (Mount Jackson, VA) for embedding in paraffin. The paraffin blocks were cut into 4-6 μm sections with a microtome and stored at RT.

In situ Hybridization Analysis

In situ hybridization (ISH) was performed using the RNAscope method (Advanced Cell Diagnostics, ACD, Newark, CA) described by Wang et al (2012). Three YS and three intestinal samples were analyzed. A set of probes for chicken PepT1 were custom synthesized by ACD. The tissue samples were processed according to the manufacturer's directions using the HybEZ oven and RNAscope 2.5 HD Detection reagent kits. All YS and small intestine samples were pretreated and hybridized as a group to minimize variation. The red and brown chromogens were used for detection of PepT1 mRNA in the YS and intestine, respectively. We found that use of the red chromogen for the YS and the brown chromogen for the intestine was optimal for visualization. Following RNAscope processing, the slides were stained with a 50% Gill #2 hematoxylin solution (Sigma Aldrich, St Louis, MO), rinsed in water, and then placed in 0.02% ammonia water. After the slides were air dried, a drop of Clear Mount solution (American Master Tech Scientific, Inc, Lodi, CA) was added and then a coverslip was placed on top. Images were captured at various magnifications with a Nikon Eclipse 80i microscope and DS-Ri1 digital camera.

Measurement of Villi Length and Statistical Analysis

The intestinal villus lengths were measured from the top of the intestinal crypt to the tip of the villi after ISH using Infinity Analyze imaging software (Lumenera Corporation, Ottawa,

Ontario). Ten structurally intact villi were randomly chosen to measure the villi lengths in the duodenum, jejunum, and ileum from e19 to D7. All data were analyzed by t-test and further compared by Tukey's test using JMP Statistical Discovery Software v10.0 (SAS Institute Inc., Cary, NC).

RESULTS

In order to assess embryo growth during incubation, the yolk-free body weights of embryos were measured. The embryo weights were 2.02±0.2 g (e11), 6.61±0.39 g (e13), 15.02±2.05 g (e15), 20.28±1.02 g (e17), 27.23±2.35 g (e19) and 38.36±2.90 g (DOH).

In situ hybridization was performed to study the spatial distribution of PepT1 mRNA expression in the YS and small intestine. Three replicates were analyzed for both the YS and small intestine. Because the replicates showed a similar pattern of hybridization, only representative images of the YS at e11, e13, e15, e17, e19 and DOH are shown in Figure 4.1. PepT1 mRNA (red chromogen) was barely detectable at e11, but was expressed strongly at e13, e15, and e17. At e19 there was decreased expression of PepT1 mRNA and by DOH expression was again barely detectable. Expression of PepT1 mRNA in the YS was localized to the small cytoplasmic region of the epithelial cells that surrounds the large lipid drop.

Images of PepT1 mRNA expression in the duodenum, jejunum, and ileum at e19, DOH and D1, D4, and D7 post hatch are shown in Figure 4.2. At e19, PepT1 mRNA was barely detectable in the small intestine. At DOH, D1, D4 and D7 there was strong expression of PepT1 mRNA in all three segments of the small intestine. At DOH and D1, cells expressing PepT1 mRNA were

localized from part way up the intestinal villi to the tip of the villi in the duodenum and jejunum. However, at D4 and D7, PepT1 mRNA was detected in cells right above the crypts but was only lightly if at all expressed at the villus tips. The spatial distribution of PepT1 mRNA in the ileum was consistently localized in cells part way up the villi to the tip of the villi from DOH to D7. Qualitatively, there was more intense staining for PepT1 mRNA in the duodenum compared to the jejunum and ileum within a sampling day. There was no staining for PepT1 mRNA in the intestinal crypts.

The lengths of the villi in the duodenum, jejunum, and ileum from e19 to D7 are shown in Figure 4.3. The villi lengths varied with age. In the duodenum villi lengths increased from e19 to D7; whereas in the jejunum and ileum villi lengths increased from DOH to D7 and D1 to D7, respectively. Villi lengths also differed between intestinal segments at the various ages. At e19, there was no difference between the villi lengths in the three intestinal segments. At DOH villi length in duodenum was greater than the jejunum and ileum. At D1 to D7, the villi were the longest in the duodenum, intermediate in the jejunum and shortest in the ileum.

DISCUSSION

In this study, ISH was used to determine the profile of PepT1 mRNA in the chicken YS during embryogenesis and the small intestine during the late embryonic and early posthatch periods. The advantage of using ISH to profile gene expression compared to qPCR is the ability to identify specific cells expressing PepT1 mRNA. As expected, although not previously shown for chicken, PepT1 mRNA was localized to a population of absorptive epithelial cells in contact with the yolk in the YS and in contact with the lumen along the intestinal villi.

Our results showed that there were temporal changes in the expression of PepT1 mRNA in the YS. Mobbs and McMillan (1979, 1981) showed that endodermal epithelium in the area vasculosa of the YS are packed with lipid drops. We found staining for PepT1 mRNA localized in the small amount of cytoplasm on the basal side of these epithelial cells. PepT1 mRNA was highly expressed in the YS from e13 to e17, indicating that this was the period during embryogenesis for maximal peptide uptake from the yolk. These ISH results are consistent with results for PepT1 mRNA expression in the YS quantified by qPCR, which showed peak PepT1 mRNA expression between e13 and e17 (Yadgary et al., 2011; Speier et al., 2012). The YS also expressed peptidases and amino acid transporters. A similar pattern of increase in the mRNA abundance of aminopeptidase N, which cleaves amino acids from the amino terminus of proteins, was observed, which showed peak expression around e15 to e17 that then decreased to DOH. Amino acid transporters that were expressed in the YS included the neutral amino acid transporter B^oAT, which increased from e11 to e19 and then declined and the anionic amino acid transporter EAAT3, which increased from e11 to DOH (Yadgary et al., 2011; Speier et al., 2012). Other amino acid transporters were expressed in the YS but have not yet been profiled in detail (Yadgary et al., 2014).

During the period between e13 and e17, Yadgary et al. (2010) showed that the weight of the yolk sac (denoted as the yolk sac membrane, YSM) increased by approximately 50% and then declined after e17. In addition, embryo weight increased 2.7 to 2.9-fold from e13 to e17, depending upon the age of the hen flock. We observed a similar 3.1-fold increase in embryo weight from e13 to e17. Thus, the uptake of amino acids by PepT1 and other amino acid

transporters contributed to the increase in weight of the YS as well as the embryo. Yadgary et al. (2010) did not observe a decrease in the protein content of the yolk during this time and attributed this to the influx of proteins into the yolk from other egg compartments such as the albumen or the amnion.

In the small intestine, PepT1 mRNA was expressed in a tissue- and development specific manner. A number of specialized cells line the villi including absorptive enterocytes and secretory goblet, Paneth and enteroendocrine cells, while the stem cells reside in the crypts (Carulli et al., 2014). Staining for PepT1 mRNA was localized to the absorptive enterocytes along the villi and was not observed in the intestinal crypts, which are the presumed sites for the stem cells. PepT1 mRNA, detected by ISH, was low at e19 in each part of the small intestine and was sharply upregulated at DOH. These results are consistent with the qPCR results that showed a rapid rise of PepT1 mRNA in the intestine between e19 and DOH (Gilbert et al., 2007; Speier et al., 2012; Zwarycz and Wong, 2013).

Abundance of PepT1 mRNA posthatch, as quantified by qPCR showed an increase from DOH to D14 or D21 (Gilbert et al., 2007; Miska et al., 2015). For our ISH results, it is more difficult to quantify expression based on staining intensity. No clear differences in staining intensity of the absorptive cells along the villi were observed between DOH and D7. Because the lengths of the villi increased with age in the duodenum, jejunum, and ileum, from D1 to D7, this likely contributed to the increase in PepT1 mRNA abundance reported by qPCR. The difference in villi lengths between the three intestinal segments was consistent with previous reports (Geyra et al., 2001; Yamauchi, 2002). In addition, we found that the timing of the increase in villi lengths was

different for each intestinal segment. The villus length of the duodenum increased significantly from e19 to D7. However, the jejunal villus length increased starting from DOH, and the ileum increased starting from D1. This result was similar to the change in the length of the small intestinal villi reported by Uni et al. (1999).

Differences in the pattern of PepT1 mRNA expression between the intestinal segments from DOH to D7 were observed. At DOH and D1, epithelial cells expressing PepT1were present from the tip of the villus to part way down the villus. This was especially evident in the duodenum, where the duodenal villi were longer than the jejunal and ileal villi. Staining was never observed in the crypts, which indicated that the stem cells did not express PepT1. The non PepT1 expressing cells above the crypts likely represent transit-amplifying (TA) cells. Transit amplifying cells are a population of progenitor cells that arise from stem cells and differentiate into absorptive and secretory cells (Carulli et al., 2014). These proliferating cells likely contribute to the rapid growth of the villi during the early posthatch period. Because the TA cells have not started differentiating, they do not express PepT1 characteristic of an absorptive enterocyte. At D4 and D7, PepT1 expressing absorptive enterocytes were detected just above the crypt, which suggested that there were fewer TA cells that had not initiated a differentiation program.

One intriguing finding was the lack of PepT1 expressing cells at the tips of the villi in the duodenum and jejunum at D4 and D7. Cells at the tip of the villi are sloughed off into the lumen as the villus grows and goes through the normal process of renewal. Gavrieli et al. (1992), showed that in the rodent small intestine, the cells located at the tips of the villi were found to

degrade and shed into the intestinal lumen. Our ISH results indicated that the cells at the tip of the duodenum and ileum may no longer be functional absorptive cells, although it is possible that PepT1 protein may still be present and functional in the brush border membrane even in the absence of PepT1 mRNA. In the ileum at D4 and D7, expression of PepT1 mRNA was still found in the cells at the tip of the villi. The difference in PepT1 mRNA expression in the cells at the tip of the villus in the duodenum and jejunum versus the ileum cannot be attributed to simply the length of the villi. Although we observed that the duodenal and jejunal villi were approximately 2-2.5-fold longer than the ileal villi at D7, Geyra et al (2001) showed that the migration rates of enterocytes along the duodenum and jejunum was approximately 3-fold faster than that of enterocytes along the ileum. Thus, the transit time for enterocytes to migrate from the crypt to the tip of the villus would be approximately the same in the duodenum, jejunum, and ileum. Uni et al. (2000) determined using BrdU labeling that in 2 day old chickens the transit time for enterocytes from crypt to villus tip was 72 hours.

In summary, we found that expression of PepT1 mRNA in the absorptive cells of the YS peaked between e13 and e17. In the intestine, PepT1 rapidly increased between e19 and DOH. There were differences in the spatial distribution of the enterocytes expressing PepT1 in the villi. In the duodenum and jejunum at DOH and D1, enterocytes expressing PepT1 mRNA were located from the tip of the villus to part way down the villi. Cells in the crypt and adjacent to the crypt did not express PepT1 mRNA, which presumably are stem cells and transit-amplifying cells, respectively. At D4 and D7, enterocytes in the duodenum and jejunum that express PepT1 mRNA were present along the length of the villi with the exception of the tip, which suggested that the cells in the process of being sloughed off may no longer be functional intestinal

enterocytes. These studies provide a more detailed profile of the ontogeny of PepT1-expressing absorptive cells in the chicken small intestine and YS.

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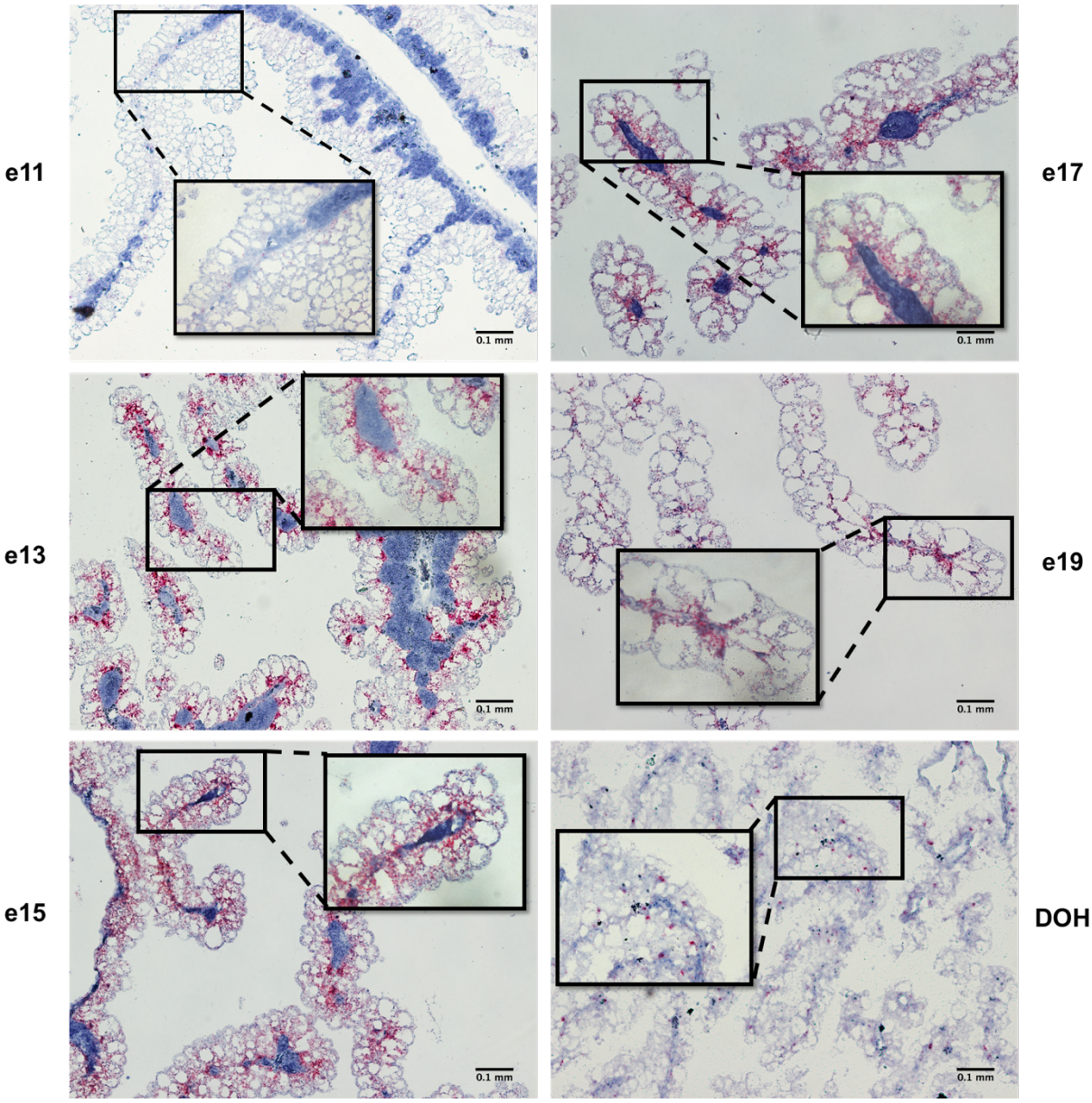

Figure 4.1 Expression profiles of PepT1 mRNA in the yolk sac during embryogenesis. Yolk sac samples from the area vasculosa were fixed in formalin and embedded in paraffin. Expression of PepT1 mRNA in cells was assayed by *in situ* hybridization using the RNAscope 2.5 HD kit (Red). The red dots showed the location of PepT1 mRNA. The tissues were counterstained with 50% hematoxylin. Images were captured using 100x magnification with the picture inserts showing 400x magnification. The scale bar represents 0.1 mm.

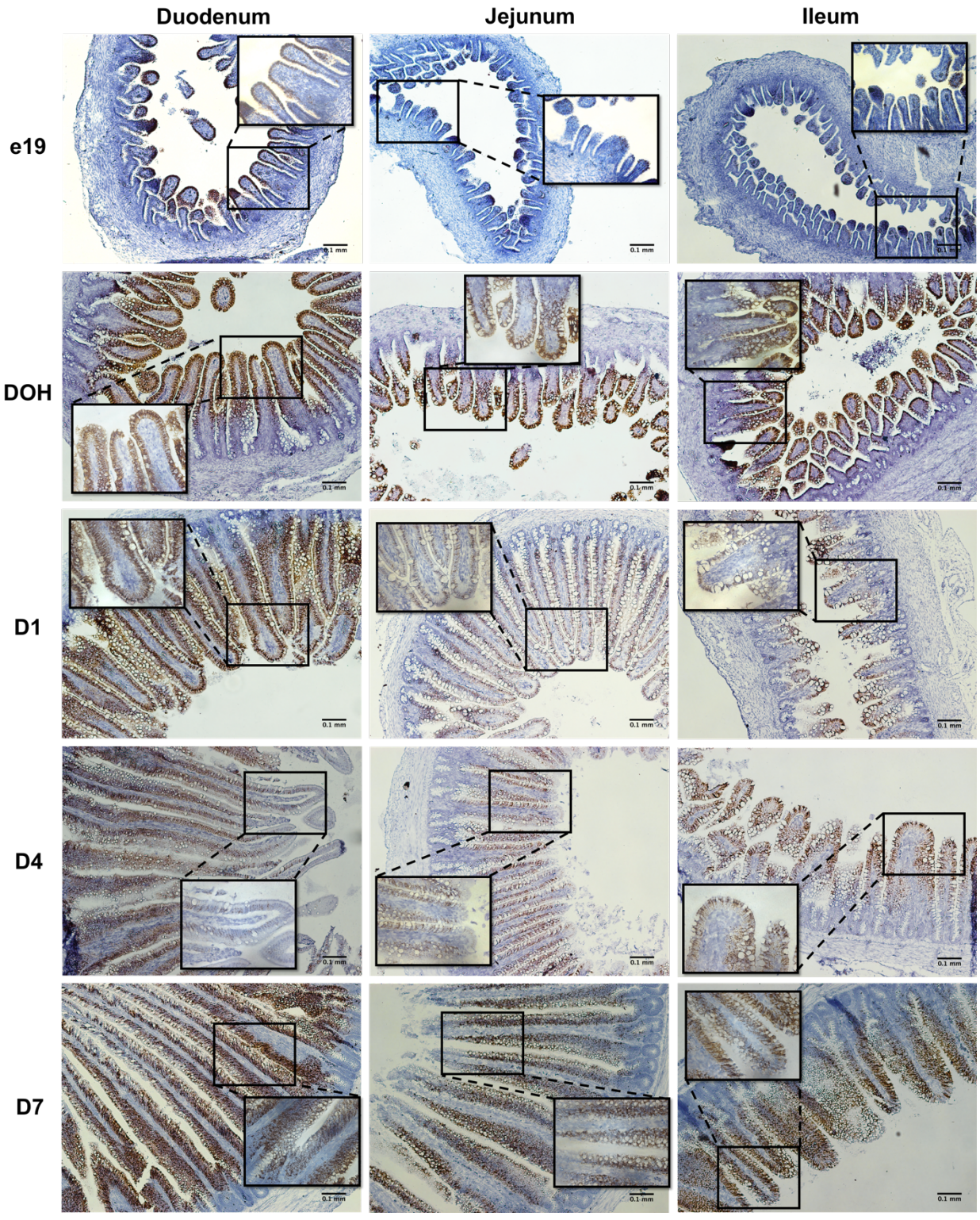

Figure 4.2 Expression profiles of PepT1 mRNA in intestine during late embryogenesis and post hatch. Intestinal samples were fixed in formalin and embedded in paraffin. Expression of PepT1

mRNA in cells was assayed by *in situ* hybridization using the RNAscope 2.5 HD kit (Brown). The brown dots showed the location of PepT1 mRNA. The tissues were counterstained with 50% hematoxylin. Images were captured using 100x magnification with the picture inserts showing 400x magnification. The scale bar represents 0.1 mm.

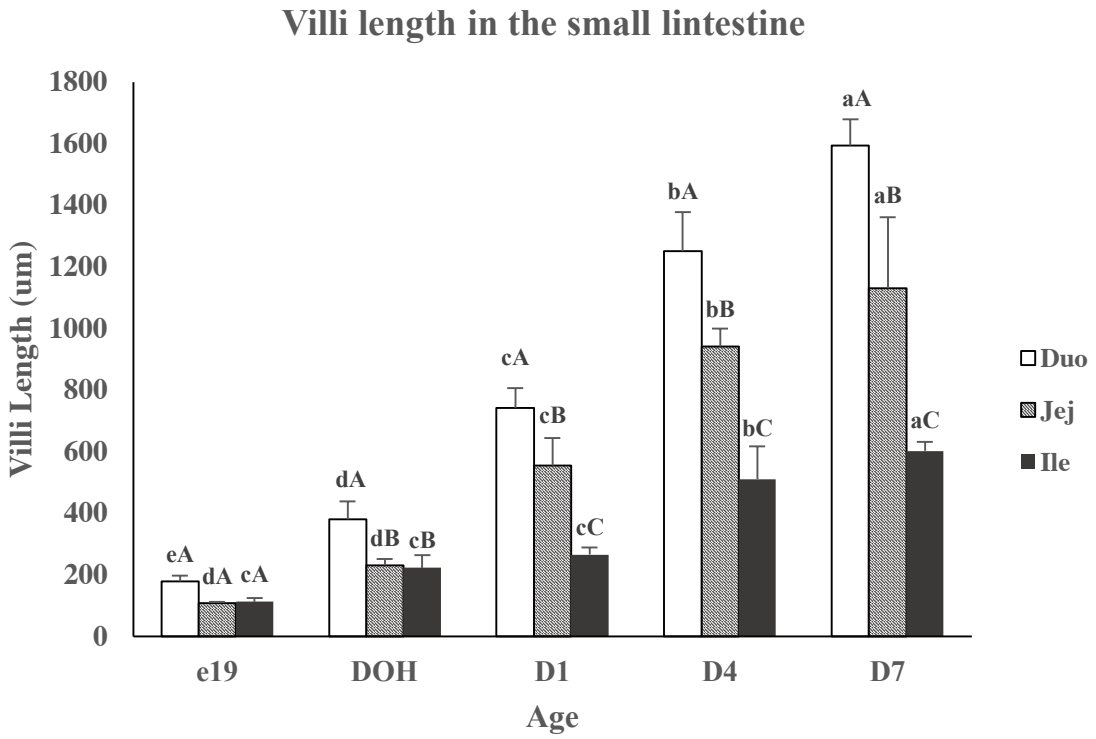

Figure 4.3 Villi lengths in different parts of the small intestine from e19 to D7. The uppercase letters on each bar indicate significant differences (P<0.05) between duodenum (duo), jejunum (jej) and ileum (ile) within a day. The lowercase letters indicate significant differences (P<0.05) between different days for a specific intestinal segment.

Chapter V

Identification of cells expressing *OLFM4* and *LGR5* mRNA by in situ hybridization in the yolk sac and small intestine of embryonic and early posthatch chicks

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ABSTRACT

The chicken yolk sac (YS) and small intestine are essential for nutrient absorption during the prehatch and posthatch periods, respectively. Absorptive enterocytes and secretory cells line the intestinal villi and originate from stem cells located in the intestinal crypts. Similarly, in the YS there are absorptive and secretory cells that presumably originate from a stem cell population. Leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) and olfactomedin 4 (Olfm4) are two widely used markers for intestinal stem cells. The objective of this study was to map the distribution of putative stem cells expressing *LGR5* and *OLFM4* mRNA in the chicken small intestine from the late embryonic period to early posthatch and the YS during embryogenesis. At embryonic days 11, 13, 15, 17 and 19 the YS was collected (n=3) and small intestine was collected at embryonic day 19, day of hatch (doh) and days 1, 4 and 7 posthatch (n=3). Cells expressing *OLFM4* and *LGR5* mRNA were identified by *in situ* hybridization. In the YS, cells expressing only *LGR5* and not *OLFM4* mRNA were localized to the vascular endothelial cells lining the blood vessels. In the small intestine, cells in the intestinal crypt expressed both *LGR5* and *OLFM4* mRNA. Staining for *OLFM4* mRNA was more intense than *LGR5* mRNA, demonstrating that Olfm4 is a more robust marker for stem cells than Lgr5. At embryonic day

19 and doh, cells staining for *OLFM4* mRNA were already present in the rudimentary crypts, with the greatest staining in the duodenal crypts. The intensity of *OLFM4* mRNA staining increased from doh to d7 posthatch. Dual label staining at doh for the peptide transporter PepT1 and Olfm4 revealed a population of cells above the crypts that did not express Olfm4 or PepT1 mRNA. These cells are likely progenitor transit amplifying cells. Thus, avians and mammals share similarity in the ontogeny of stem cells in the intestinal crypts.

Key words: stem cells, Lgr5, Olfm4, yolk sac, small intestine

INTRODUCTION

During incubation, the developing chick embryo is dependent on the yolk and other egg contents for its nutrients. These nutrients, which are absorbed through the yolk sac (YS), are critical for embryonic growth and development. In the first week of incubation, endodermal cells advance from the developing gut of the embryo and gradually spread over the surface of the yolk (Patten, 1971; Mobbs and McMillan, 1979). Thus, the intestine and YS form a contiguous tissue. Concomitant with the increase in endodermal area, mesodermal and ectodermal layers also form, generating three distinct regions of the YS known as the area pellucida, area vasculosa and area vitellina. In the area vasculosa, columnar endodermal cells are responsible for nutrient digestion and absorption while mesodermal cells are important for hematopoiesis and vasculogenesis (Sheng, 2010). Around embryonic day 15, the area vasculosa shows upregulation of nutrient transporters (Yadgary et al., 2011, 2014; Speier et al., 2012). At the end of the embryonic period, the YS begins to degrade as the yolk is drawn into the abdominal cavity.

CHAPTER V. OLFM4 AND LGR5 IN SMALL INTESTINE AND YOLK SAC

During the posthatch period, the chicken gastrointestinal tract plays a critical role for nutrient metabolism. The small intestine is a major site for secretion of digestive enzymes and endocrine hormones and for nutrient absorption. The multiple functions of the small intestine are due to the combined actions of different cell types along the small intestinal villi. In contrast to the YS, the various functions of each epithelial cell type in the small intestine have been described in detail. In the mammalian model, epithelial cells of the villus originate from stem cells located in the intestinal crypt (reviewed in Clevers, 2013; Barker, 2014; Carulli et al., 2014). Intestinal stem cells can divide to generate either more stem cells or a population of cells that will become differentiated cells, such as enterocytes, enteroendocrine cells, Paneth cells, and goblet cells. The majority of the cells along the intestinal villi are enterocytes, which are associated with digestive and absorptive functions. Paneth cells are located in the intestinal crypt rather than along the intestinal villi. There are also proliferating transit-amplifying (TA) cells located between the intestinal crypt cells and mature enterocytes, which serve as intermediate progenitor cells.

Leucine-rich repeat containing G protein-coupled receptor 5 (Lgr5) is an important intestinal stem cell marker involved in the Wnt signaling pathway, which is important in mediating the differentiation of intestinal stem cells (Haegebarth and Clevers, 2009). Different from other Wnt related genes, Lgr5 is expressed exclusively in the intestinal stem cells, but not in Paneth cells in the crypt (Barker et al., 2007). Lgr5 also has been found to be expressed in the cycling stem cells in adult stomach, intestine, hair follicle, and some mammalian glands (Jaks et al., 2008; Vries et al., 2010; Kemper et al., 2012; Wu et al., 2013), which indicates its global stem cell function during the postnatal stage. Recently, Lgr5 was shown to be associated with hematopoiesis in fetal and adult livers, suggesting that Lgr5 was a marker for hematopoietic stem

cells (Liu et al., 2014). The relatively low expression of Lgr5, however, limits its use. Olfactomedin 4 (Olfm4) is an intestinal stem cell marker that is strictly expressed in the intestinal crypt at a much greater level than Lgr5 and thus is a more robust intestinal stem cell marker (Schuijers et al., 2014). Olfm4 is a secreted protein that is involved in many cell signaling pathways related to cell proliferation, regeneration and apoptosis (Liu and Rodgers, 2016).

Although much is known about stem cells in the mammalian small intestine, little is known about stem cells in the intestine and YS of chickens. The objective of this project was to identify and localize by *in situ* hybridization putative stem cells expressing *LGR5* and *OLFM4* mRNA in the YS and intestine during embryogenesis and the early posthatch period.

MATERIALS AND METHODS

Animals and Tissue Collection and Processing

Cobb 500 eggs were obtained from a local hatchery (Cobb Vantress, Wadesboro, NC) transported to Virginia Tech and incubated at 38.5ºC. All animal procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. On embryonic day 11 (e11), e13, e15, e17, and e19, eggs were opened and embryos were killed by cervical dislocation. The YS (n=3) was collected at these days and intestine (n=3) was collected at only e19. At day of hatch (doh), chicks were killed by cervical dislocation and the YS and intestine were collected (n=3). The remainder of the chicks were placed into a pen with ad libitum access to water and a standard corn-soybean starter diet. At d1, d4, and d7 posthatch, small intestine was collected from chicks (n=3).

For tissue processing, the YS was removed and rinsed in phosphate buffered saline (PBS) to remove yolk contents and a sample was collected from the area vasculosa. The intestine was separated into duodenum, jejunum, and ileum and then rinsed in PBS to remove the intestinal contents. The area vasculosa and small intestinal segments were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific, Waltham, MA) for one day and then stored in 70% ethanol prior to embedding in paraffin (Histo-Scientific Research Labs Inc., Mount Jackson, VA). Sections (4-6 μm) were cut with a microtome (Microm HM 355S, Thermo Fisher Scientific, Waltham, MA).

In situ Hybridization Analysis

In situ hybridization (ISH) was performed using the RNAscope (Advanced Cell Diagnostics, ACD, Newark, CA) method as described by Wang et al. (2012). Singleplex probes for *OLFM4* and *LGR5* mRNA and multiplex probes for the peptide transporter *PEPT1* and *OLFM4* mRNA were custom designed by ACD. For intestinal tissue, RNAscope assays using singleplex probes or multiplex probes for dual labeling were performed with the RNAscope 2.5 HD Assay-BROWN or RNAscope 2.5 HD Duplex Assay, respectively, following the manufacturer's instructions. After processing, slides were stained for 2 minutes with 50% Gill #2 hematoxylin solution (Sigma Aldrich, St Louis, MO), rinsed in distilled water and placed in 0.02% ammonia water until the stain turned from purple to blue. After the slides were dehydrated in a graded series of ethanol (70%, 95%, 95%) for 2 min each followed by xylene for 5 minutes, the samples were air dried. A few drops of Clear Mount solution (American Master Tech Scientific, Inc, Lodi, CA) were placed on the tissue and sealed with a coverslip. Images

were captured under bright field with a Nikon Eclipse 80i microscope and DS- 119 Ri1 digital camera.

For the YS, singleplex probes were used with the RNAscope 2.5 HD Assay-RED, because the red chromogen is more sensitive and better for detection of low abundance mRNAs. The manufacturer's instructions were followed with the following modifications. Because the YS is a thin, fragile membrane, the time that the sample was boiled during target retrieval was reduced from 15 minutes to 5 minutes. After hematoxylin treatment, the samples were dried at 60ºC prior to dehydration in xylene. The graded series of ethanol step was omitted.

RESULTS

In situ hybridization (ISH) was used to identify cells that express *OLFM4* and *LGR5* mRNA. Representative images of cells expressing *OLFM4* mRNA (brown staining) in the duodenum, jejunum, and ileum at e19, doh, d1, d4, and d7 posthatch are shown in Figure 5.1A. At e19, *OLFM4* mRNA was detected in cells at the base of the intestinal villi in the duodenum and jejunum, which is the site of the developing crypts. In the ileum, only a few cells at the base of the villi expressed *OLFM4* mRNA. Qualitatively, at e19 the number of cells expressing *OLFM4* mRNA in the crypt was greatest in the duodenum, moderate in the jejunum, and lowest in the ileum. In addition, there was an increase in intensity of staining for *OLFM4* mRNA with developmental age from doh until d7. There was no obvious difference in *OLFM4* mRNA staining between intestinal segments at d4 and d7.

Expression of *LGR5* mRNA in duodenum, jejunum, and ileum at e19, doh, d1, d4, and d7 posthatch is shown in Figure 5.1B. The red chromogen was used for *in situ* hybridization because of its increased sensitivity. The pattern for *LGR5* mRNA staining was similar as that for

OLFM4 mRNA (Figure 5.1A). Cells expressing *LGR5* mRNA were located in the intestinal crypts and increased from e19 to d7. There was less intense staining for *LGR5* mRNA than *OLFM4* mRNA.

Dual label *in situ* hybridization for *OLFM4* and the peptide transporter *PEPT1* mRNA in the chicken small intestine at doh is shown in Figure 5.2. The *PEPT1* mRNA expressing cells are distributed from the middle to the tip of the intestinal villus (blue-green staining) and mark absorptive cells, while *OLFM4* mRNA expressing cells are localized in the intestinal crypts (red staining). There were cells located above the crypts that did not express *PEPT1* or *OLFM4* mRNA. These cells are likely the intermediate progenitor TA cells. This was most obvious in the duodenum and jejunum and less apparent in the ileum. In addition, the mucin producing goblet cells are clearly identified as non-PepT1 expressing cells along the intestinal villi.

The YS is a multi-functional organ that contains a variety of differentiated cells. The location of putative stem cells expressing *LGR5* or *OLFM4* mRNA was determined for e11 to e19. There were no cells staining for *OLFM4* mRNA in the YS (data not shown). Cells staining for *LGR5* mRNA (red staining) were present among the vascular endothelial cells lining the blood vessels from e11 until e17 (Figure 5.3). At e19, as the YS was beginning to degrade, cells expressing *LGR5* mRNA became focused into small clusters of cells surrounding the collapsing blood vessels.

DISCUSSION

Because the intestine and YS form a contiguous structure and serve a similar function for nutrient uptake, similarities or differences in expression of the intestinal stem cell marker genes *OLFM4* and *LGR5* were investigated in both tissues. In the YS, there was no staining for *OLFM4*

mRNA, while staining for *LGR5* mRNA was localized to the vascular endothelial cells surrounding the blood vessels. Nagai and Sheng (2008) used a beta A globin-specific probe to mark definitive erythrocytes and showed staining of clusters of cells adjacent to arteries associated with venous vessels in the YS. They concluded that one source of definitive erythrocytes was the existing endothelial vascular cells. Samokhvalov et al. (2007) showed that YS cells that express Runx1, which is an important gene for establishment of the definitive erythropoietic system, colonize the aorta-gonad-mesonephros region. Liu et al. (2014) reported that Lgr5 was able to mark hematopoietic stem and progenitor cells in the aorta-gonad-mesonephros region of mouse embryos. Thus, it is possible that the vascular endothelial cells expressing *LGR5* mRNA in the chicken YS are also hematopoietic stem cells.

The YS contains a number of differentiated cells that are important for the uptake of yolk macromolecules such as fat, amino acids and sugars (Bauer et al., 2013; Yadgary et al., 2014). Using *in situ* hybridization, Zhang and Wong (2017) identified absorptive epithelial cells in the YS that express mRNA for the peptide transporter PepT1. Recently, transcriptome analyses of the YS in human, mouse, and chicken revealed that the nutrient absorption function of the YS epithelium is conserved in different species (Cindrova-Davies et al., 2017). Thus, the ontogeny of these absorptive cells in various species may be similar, however it has not yet been shown that $Lgr5^{+}$ cells are precursors for absorptive cells in chickens.

At doh, the small intestinal crypts are rudimentary and contain only a few cells (Uni et al., 2000). We observed cells expressing *OLFM4* mRNA at the base of the villi at e19, with greater staining for *OLFM4* mRNA in the duodenum than ileum. At this age, the intestinal crypts have not formed; however, cells that express *OLFM4* mRNA and are presumably intestinal stem cells are already present. The number of cells expressing *OLFM4* mRNA increased from e19 to

doh as the crypts further developed. Although the crypts are rudimentary at doh, putative stem cells expressing *OLFM4* mRNA are already present.

The pattern of *LGR5* and *OLFM4* mRNA expressing cells was similar at d7 with both markers identifying cells in the crypt. There was more intense staining for *OLFM4* than *LGR5* mRNA. This result is consistent with the ISH results for *LGR5* and *OLFM4* mRNA in the mouse small intestine (Schuijers et al., 2014). These results demonstrate that Olfm4 is a more robust marker for intestinal stem cells than Lgr5 in both mammals and avians. In mammals, Lgr5 marks stem cells in tissues other than intestine such as stomach, intestine, hair follicle and tongue (Barker et al., 2007; Jaks, et al., 2008; Kemper et al., 2012; Yee et al., 2013), while Olfm4 plays multiple roles in innate immunity, inflammation and cancer (Liu and Rodgers., 2016). Qualitatively, there was an increase in the number of cells expressing *OLFM4* mRNA from d1 to d4, which would coincide with the time that there was an increase in villus area, villus height and crypt depth (Uni et al., 1999). During this time, an active population of stem cells would be needed to supply enterocytes for villus elongation.

Based on the mammalian model developed from rodent studies, Paneth cells are interspersed with stem cells in the crypts (Clevers, 2013; Barker, 2014; Carulli et al., 2014). Paneth cells produce antimicrobial products such as lysozyme and cryptdins/defensins and are important for development of Lgr5-expressing stem cells (Sato et al., 2011). Paneth cells, however, do not express *LGR5* mRNA and thus there is a clear alternating pattern of $Lgr5^{+}$ stem cells and $Lgr5^{-}$ Paneth cells in the intestinal crypts (Barker et al., 2007; Sato et al., 2011; Clevers, 2013). In our chicken study, all cells in the crypt expressed *LGR5* or *OLFM4* mRNA suggesting the absence of Paneth cells that do not express *LGR5* mRNA. The existence of Paneth cells in the chicken remains controversial. Nile et al. (2004) showed that there was mRNA expression of

lysozyme c, which is a widely used marker of Paneth cells, in 5 d old chicks but not in 17 or 38 d old chicks, suggesting the absence of Paneth cells in older chickens. In contrast, Wang et al. (2016) showed by electron microscopy and staining with phloxine-tartrazine that cells in the intestinal crypts contained granules, which are characteristic of Paneth cells. In addition, cells in the crypt were shown by *in situ* hybridization to express mRNA for lysozyme c.

The identification of cells expressing *PEPT1* or *OLFM4* mRNA in the small intestine revealed a population of cells located between stem cells in the crypts and enterocytes along the villus, which expressed neither *PEPT1* nor *OLFM4* mRNA. Based on the mammalian villus/crypt model, these cells are likely TA cells, which are a population of intermediate progenitor cells that arise from stem cells and later differentiate into specific cell types (Carulli et al., 2014). This suggests that chicken and mammals share the same pathway from stem cell to progenitor TA cells to mature absorptive cells. Uni et al. (2000) previously identified proliferating cells in the intestinal villi of posthatch chickens using PCNA (proliferating cell nuclear antigen) and BrdU (5-bromo-2-deoxyuridine) assays. They found proliferating cells that were located along the intestinal villi and crypts around 2 hours posthatch, which gradually migrated to the tip of the intestinal villi at day 1 posthatch. Thus, the TA cells we identified likely represent only one population of proliferating cells in the chicken intestinal villus/crypt.

In summary, expression of *LGR5* but not *OLFM4* mRNA in the YS was localized to endothelial cells that line the blood vessels, which may be hematopoietic stem cells. In contrast, cells in the intestinal crypts expressed both *LGR5* and *OLFM4* mRNA, with Olfm4 acting as a more robust marker than Lgr5. Different from mammals, all cells in the intestinal crypt of chickens expressed *LGR5* and *OLFM4* mRNA, indicating the absence of Paneth cells that express neither gene. In addition, there was a population of progenitor TA cells that were located

between the Olfm4/Lgr5 expressing crypt cells and the PepT1 expressing absorptive cells and expressed neither *OLFM4* nor *LGR5* mRNA. Thus, there are similarities and differences between the organization and development of cells in the mammalian and chicken villus and crypt.

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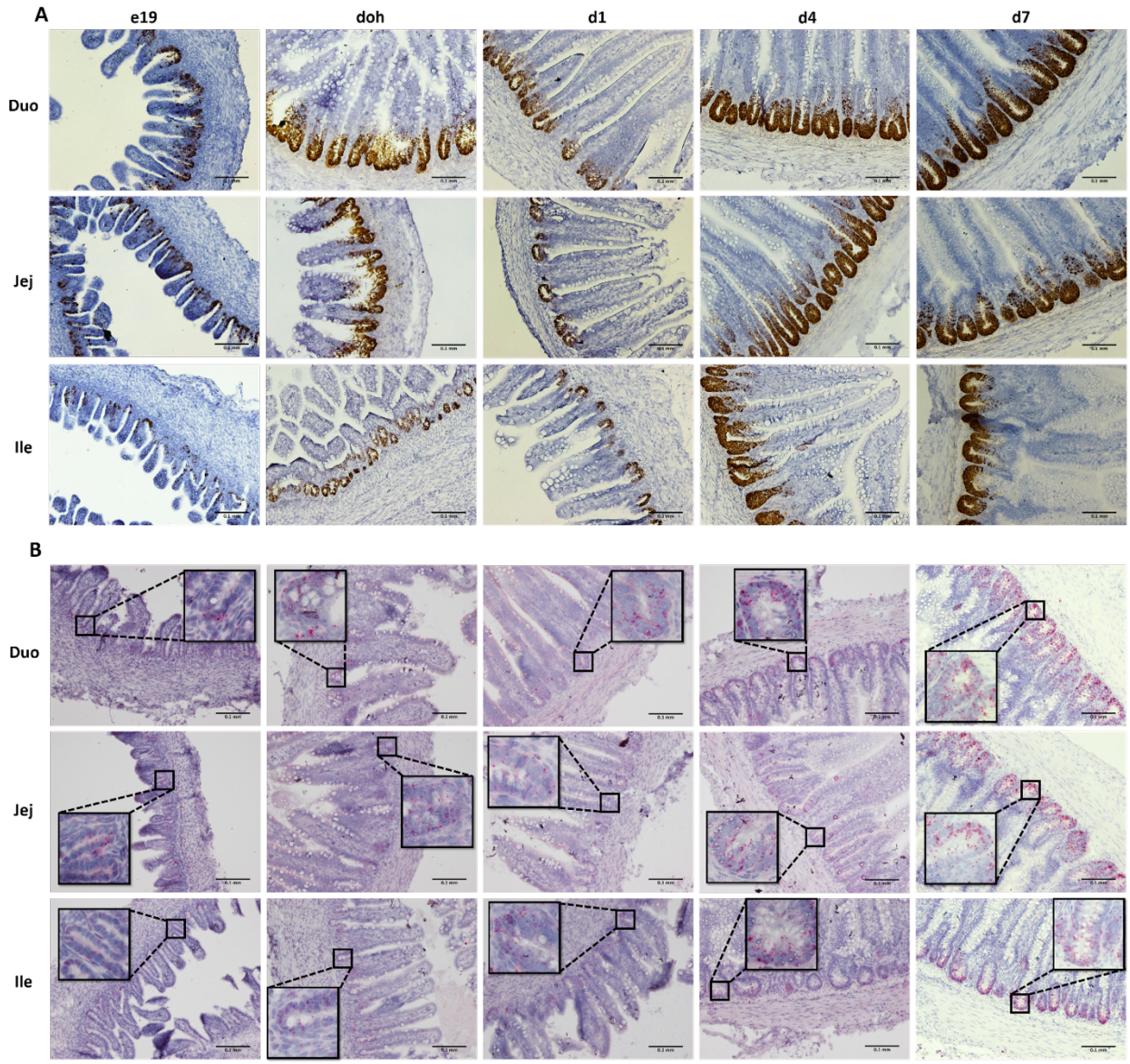

Figure 5.1 Expression of *OLFM4* and *LGR5* mRNA in the intestine from late embryogenesis to early posthatch using *in situ* hybridization. Tissue samples were collected from duodenum (Duo), jejunum (Jej) and ileum (Ile) at embryonic day 19 (e19), day of hatch (doh) and posthatch d 1 (d1), 4 (d4), and 7 (d7). Samples were fixed in formalin, embedded in paraffin and analyzed by *in situ* hybridization. A. Cells expressing *OLFM4* mRNA (brown staining) were detected using RNAscope 2.5 HD Assay-BROWN. B. Cells expressing *LGR5* mRNA (red staining) were detected using RNAscope 2.5 HD Assay-RED. Images were captured using 200x magnification.

The images in the inserts are 400x magnification. The tissues were counterstained with 50% hematoxylin. The scale bar represents 0.1 mm.

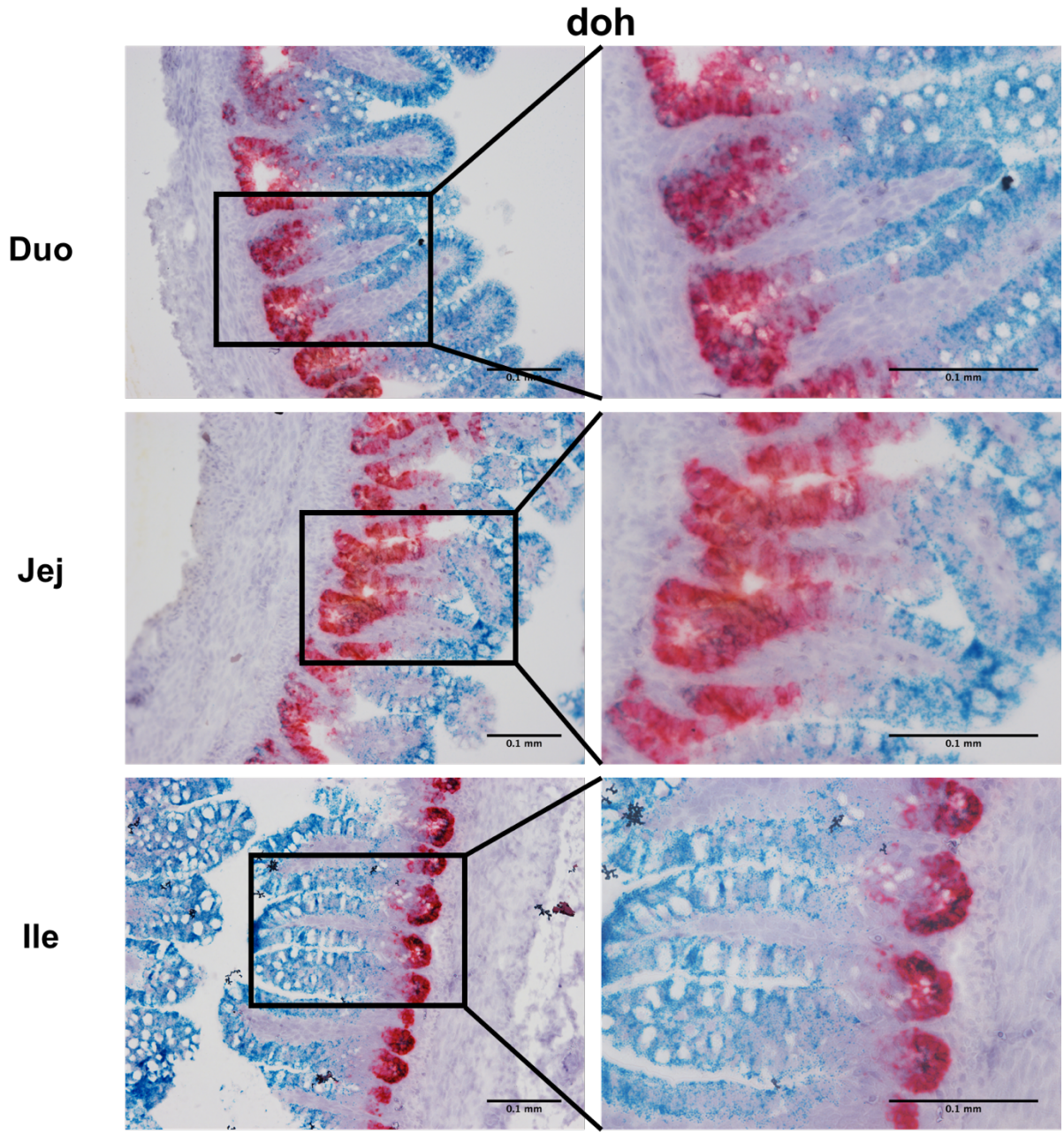

Figure 5.2 Expression of *OLFM4* and *PEPT1* mRNA in the intestine using dual label *in situ* hybridization. Tissue samples were collected from duodenum (Duo), jejunum (Jej) and ileum (Ile) at day of hatch (doh). Samples were fixed in formalin, embedded in paraffin and analyzed

by *in situ* hybridization. Cells expressing *OLFM4* mRNA (red staining) or *PEPT1* mRNA (blue-green staining) were detected using RNAscope 2.5 HD Duplex Assay. Images were captured using 200x magnification (left). The images on the right are a magnification (400x) of the area shown in the left image. The tissues were counterstained with 50% hematoxylin. The scale bar represents 0.1 mm.

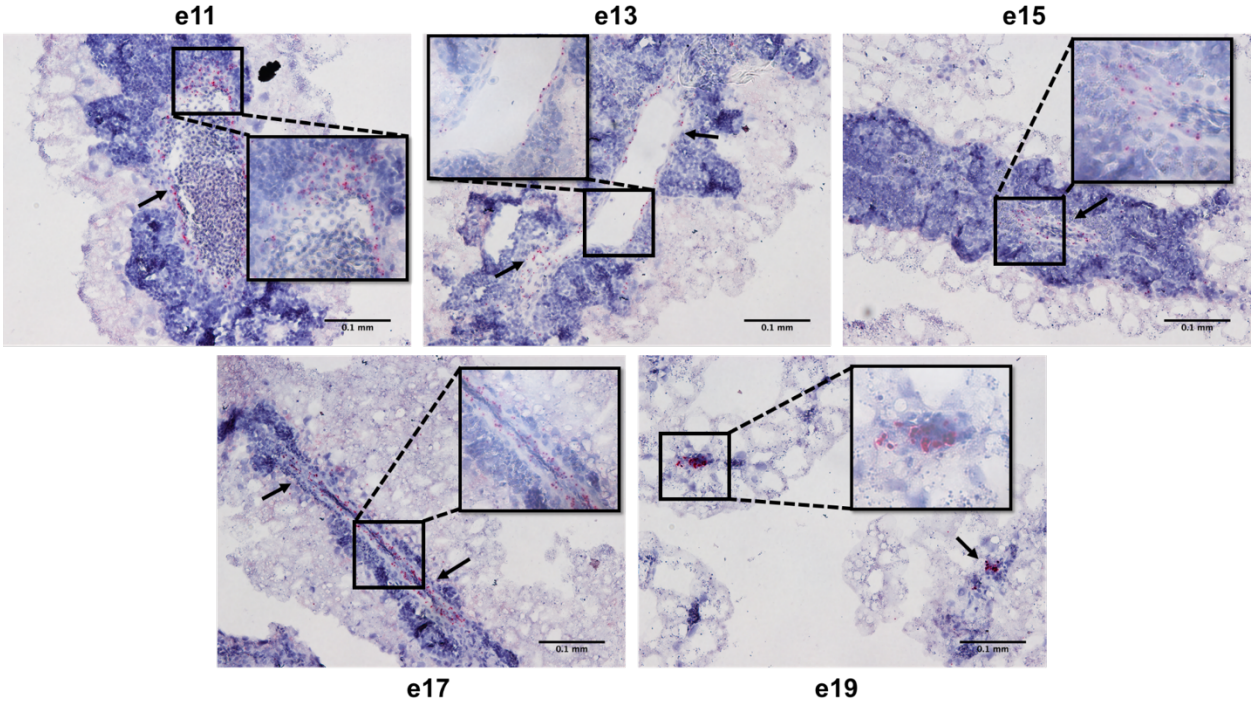

Figure 5.3 Expression of *LGR5* mRNA in the yolk sac during embryogenesis. Yolk sac samples from the area vasculosa were collected at embryonic d 11 (e11), 13 (e13), 15 (e15), 17 (e17), and 19 (e19). Tissue samples were fixed in formalin, embedded in paraffin and analyzed by *in situ* hybridization. Cells expressing *LGR5* mRNA (red staining) were detected using RNAscope 2.5 HD Assay-RED. The black arrows indicate the location of cells expressing *LGR5* mRNA. Images were captured using 200x magnification. The images in the inserts are 400x

magnification. The tissues were counterstained with 50% hematoxylin. The scale bar represents 0.1 mm.

Chapter VI

Localization of cells expressing SGLT1 mRNA in the yolk sac and small intestine of broilers

ABSTRACT

The uptake of glucose is mediated mainly by the sodium-glucose cotransporter, SGLT1. Previous studies using quantitative PCR showed that SGLT1 mRNA was induced in the yolk sac and in the small intestine prior to hatch. However, PCR analysis does not allow for the localization of cells expressing SGLT1 mRNA. The objective of this study was to use in situ hybridization to identify cells in the yolk sac and small intestine that expressed SGLT1 mRNA during the transition from late embryogenesis to early post-hatch. Expression of SGLT1 mRNA in yolk sac epithelial cells was low from embryonic day 11 to 17, peaked at embryonic day 19 and declined at day of hatch. In the small intestine, cells expressing SGLT1 mRNA were present not only along the intestinal villi but also in the crypts. There was greater expression of SGLT1 mRNA in the intestinal epithelial cells that line the villus than in the stem cells located in the crypts. The latter result suggests that stem cells have the ability to import glucose. Expression of SGLT1 mRNA in the intestine increased from embryonic day 19 to day of hatch and then maintained a high level of expression from d 1 to d 7 post-hatch. For both the yolk sac and small intestine, the temporal pattern of SGLT1 mRNA expression detected by in situ hybridization was consistent with the pattern revealed by PCR.

Key words: SGLT1, yolk sac, intestine, in situ hybridization

INTRODUCTION

From the pre-hatch to the post-hatch stage, the chick transitions from the uptake of nutrients from lipid-rich yolk by the yolk sac to uptake of nutrients from carbohydrate and protein-rich feed by the small intestine (Moran, 2007). The yolk sac develops from the embryonic gut and gradually forms the epithelial endoderm and vascular mesoderm that covers the yolk (Mobbs and McMillan, 1979; Bauer et al., 2013). The small intestine contains villi and the yolk sac contains villus-like structures that project into the yolk or intestinal lumen to increase the surface area for enhancing nutrient uptake (Holdsworth and Wilson, 1967; Mobbs and McMillan, 1981; Noble and Cocchi, 1990). Thus, the yolk sac and intestine share not only structural features but also functional properties.

The yolk sac and intestine play essential roles in the uptake of nutrients. The yolk sac is a multifunctional organ that provides the essential functions of several organs that have not yet fully developed. These functions include nutrient absorption, production of blood factors, and synthesis of blood cells (Yadgary et al., 2014). Cells of the yolk sac express several nutrient transporters that are also expressed by cells of the small intestine, such as amino acid, peptide, fat and monosaccharide transporters (Yadgary et al. 2011; Speier et al., 2012; Bauer et al., 2013; Yadgary et al., 2014; Zhang and Wong, 2017). During late embryogenesis, as the yolk sac starts to degrade, amniotic fluid is swallowed by the embryo and some yolk is transferred through the yolk stalk into the small intestine to provide nutrients that are absorbed in the small intestine (Uni et al., 2003; reviewed in Uni and Ferket, 2004). During the late embryonic and post-hatch

periods, the small intestine expresses a wide variety of nutrient transporters (Gilbert et al., 2007; Zeng et al., 2011; Miska et al., 2014; 2015).

Glucose is the major energy substrate for animals to produce ATP through aerobic oxidation. The sodium glucose transporter SGLT1 (SLC5A1) is the major glucose transporter that is located at the brush border membrane of enterocytes (Garriga et al., 1999; Barfull et al., 2002; Wright, 2013), which are derived from intestinal stem cells that are marked by olfactomedin 4 (Olfm4) and leucine rich repeat G protein coupled receptor 5 (Lgr5) in both mammals and chickens (Carulli et al., 2014; Zhang and Wong, 2018). The driving force for transport of glucose from the intestinal lumen into the enterocyte by SGLT1 is the co-transport of Na^+ down a sodium gradient, which is maintained by the Na^+-K^+ATPase on the basolateral membrane (Drozdowski and Thomson, 2006). During embryogenesis, the glucose concentration in the yolk is low and the chick embryo mainly relies on lipid oxidation to produce energy (Speake et al., 1998). Near the end of incubation (e.g., embryonic day (**e**) 19), the concentration of glucose in the yolk increases due to gluconeogenesis and glycogenolysis occurring in the yolk sac and anaerobic oxidation of glucose becomes the dominant pathway for generating energy for the embryo (Yadgary et al., 2010; Yadgary and Uni, 2012).

Studies using quantitative PCR showed that there is an increase in SGLT1 mRNA in the yolk sac and small intestine. In the yolk sac, SGLT1 mRNA showed low expression from e11 to e17, peak expression at e19 or e20, which corresponded to the increase in glucose concentration in the yolk, followed by a decrease at e21 as the yolk sac degraded (Yadgary et al., 2011; Speier et al., 2012). In the small intestine, there is an increase in SGLT1 mRNA from e19 to day of hatch, and then high-level expression post-hatch (Gilbert et al., 2007; Li et al., 2008; Speier et

al., 2012). The objective of this study was to identify by in situ hybridization, cells in the yolk sac and small intestine that express SGLT1 mRNA.

MATERIALS AND METHODS

Sample Collection and preparation

Cobb 500 eggs were obtained from a local hatchery and incubated at 37°C with 50% relative humidity. Hatched chicks were reared together in a large pen with ad libitum access to water and feed. The feed was a standard corn-soybean starter diet that was formulated to meet NRC nutrient requirements of poultry. All animal procedures were approved by the Institutional Animal Care and Use Committee at Virginia Tech. At embryonic d 11, 13, 15, 17, and 19, embryos were removed and killed by decapitation. Yolk sac (**YS**) samples (0.5 cm x 0.5 cm) were collected from the area vasculosa and rinsed in cold phosphate buffered saline (**PBS**). At e19, day of hatch (**doh**), d1, d4 and d7 post-hatch, chicks were killed by cervical dislocation and small intestinal samples were collected and separated into duodenum, jejunum, and ileum and then rinsed with cold PBS. Both yolk sac and intestinal samples were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific, Waltham, MA) for 24 hours and stored in 70% ethanol until embedding in paraffin (Histo-Scientific Research Labs Inc., Mount Jackson, VA).

In situ hybridization

The formalin fixed paraffin embedded tissues were cut into 5 μm sections using a microtome and mounted on slides. The tissue sections were deparaffinized in xylene and rinsed in absolute ethanol. In situ hybridization was performed using the RNAscope procedure

(Advanced Cell Diagnostics, Newark, CA; Wang et al., 2012). Probes to the protein coding regions of *Gallus gallus* SGLT1 (NM_001293240.1) and Olfm4 (NM_001040463.1) were custom synthesized. Probes to the bacterial gene *dapB* were supplied by ACD and used as a negative control. The RNAscope 2.5 HD Detection kit (BROWN) was used for detection of SGLT1 mRNA in the small intestine, while the RNAscope 2.5 HD Detection kit (RED) was used for detection in the yolk sac. The red chromogen in the RNAscope 2.5 HD Detection kit (RED) has the additional property of being fluorescent and thus is better for detecting low level expression. Because we expected low level SGLT1 mRNA expression in the yolk sac and high level SGLT1 mRNA expression in the intestine, we used the red chromogen for the yolk sac and the brown chromogen for the intestine. Tissue sections were first counterstained with 50% Gill #2 hematoxylin (Sigma Aldrich, St. Louis, MO) and then treated with 0.02% ammonia water to turn the stain from purple to blue. A drop of VectaMount mounting medium (Vector Laboratories, Inc. Burlingame, CA) was placed on the tissue and a coverslip was gently placed on top. Images were captured with a Nikon Eclipse 80i microscope equipped with a DS-Ri1 digital camera.

Image and Statistical Analysis

Three replicate YS samples were processed for in situ hybridization. Because each red dot represents an SGLT1 mRNA molecule, mRNA expression can be quantified. Three representative images (400X) of each YS sample were selected and the number of red dots was enumerated. The area of each YS sample that appeared on the image was measured by using Image J software developed by the National Institutes of Health (https://imagej.nih.gov/ij/). The number of dots was divided by the yolk sac sample area (mm^2) to calculate the density of SGLT1

mRNA/YS area. Data were analyzed using one-way ANOVA (α=0.05) using JMP Statistical Discovery Software v10.0 (SAS Institute Inc., Cary, NC). Significant differences were further evaluated with Tukey's test.

RESULTS AND DISCUSSION

Cells Expressing SGLT1 mRNA in the Yolk Sac

SGLT1 mRNA was detected in the yolk sac epithelial cells from e11 to doh, with the greatest expression at e19 (Fig. 6.1a). The negative control with the bacterial gene *dapB* showed no staining (Fig. 6.1a). SGLT1 mRNA expression was quantified by counting the number of dots, which represent SGLT1 mRNA molecules, per area of YS. Abundance of SGLT1 mRNA was similar between e11 and e17, peaked at e19 and then declined at doh (Fig. 6.1b), which is consistent with the staining pattern for SGLT1 mRNA shown in Fig. 6.1a. During the period of greatest SGLT1 mRNA expression (e19) and to a lesser extent at e17, SGLT1 mRNA staining appeared clustered around the nuclei of some cells, rather than dispersed throughout the cytoplasm. This may depict cells that are in the process of actively transcribing SGLT1 pre-mRNA, which has not yet been transported into the cytoplasm.

The in situ hybridization images were consistent with the results reported for qPCR analysis, which showed an increase in SGLT1 mRNA in the yolk sac from e17 to e19/e20 and then a decrease at e21 (Yadgary et al., 2011; 2014; Speier et al., 2012). The increase in SGLT1 mRNA and presumably SGLT1 transporter activity coincided with an increase in the concentration of glucose in the yolk. During embryogenesis, the amount of carbohydrate in the yolk increases, with glucose in the yolk increasing from 20 mg at e11 to 60 mg at e19 (Yadgary et al., 2010; 2012) This is partly due to the increase in mRNA expression for enzymes in the YS

that are involved in gluconeogenesis and glycogenolysis (Yadgary and Uni, 2012). Together these processes release glucose into the yolk, which can then be taken up by SGLT1 expressed in the YS epithelial cells.

Cells Expressing SGLT1 mRNA in the Small Intestine

The distribution of cells expressing SGLT1 mRNA in the duodenum, jejunum, and ileum from e19 to d 7 post-hatch is shown in Fig. 6.2. SGLT1 mRNA was expressed in not only the enterocytes lining the villi but also cells in the crypt. SGLT1 mRNA expression in the enterocytes was low at e19, increased to doh and then maintained a high level of expression from d 1 to d 7 post-hatch. SGLT1 mRNA expression in the enterocytes was qualitatively similar between duodenum, jejunum and ileum. These temporal results were consistent with the published qPCR results showing an increase in SGLT1 mRNA in chicken intestine from late embryogenesis until early post-hatch (Gilbert et al., 2007; Speier et al., 2012).

To better visualize staining for SGLT1 mRNA in cells, a higher magnification of the ends of villi are shown in Fig. 6.3, along with the negative control. SGLT1 mRNA was expressed in the epithelial cells lining the villi and did not appear to be expressed in the goblet cells, which are revealed as unstained cells. The negative control using the bacterial gene *dapB* showed no staining.

Staining for SGLT1 mRNA was also present in cells in the crypts, but at a lower level compared to the enterocytes along the villi. Similar to the enterocytes, there was an increase in staining for SGLT1 mRNA in the crypts with age, although not as dramatic as in the enterocytes of the villi (Fig. 6.2). Because the crypt contains putative stem cells that express Olfm4 in chickens (Zhang and Wong, 2018), serial sections from jejunum at d4 were stained for SGLT1

and Olfm4 mRNA and are shown at higher magnification in Fig. 6.4. Enterocytes lining the villi and cells in the crypt expressed SGLT1 mRNA, whereas only cells in the crypt expressed Olfm4 mRNA. The expression of SGLT1 and Olfm4 mRNA in crypt cells suggests that glucose is an important nutrient for proliferation of stem cells. Zhou et al. (2018) showed that the addition of glucose to mouse intestinal epithelial crypts increased crypt proliferation and glycolysis.

A number of studies have examined the localization of SGLT1 mRNA expression using in situ hybridization and of SGLT1 protein using immunocytochemistry in different species with varying results. SGLT1 mRNA was expressed in the epithelial cells along the villus but not in the crypts of the small intestine of rabbits (Hwang et al., 1991) and mice (Yoshikawa et al., 2011). Consistent with the mRNA results, SGLT1 protein was detected only in the brush border membrane of the enterocytes along the villus and not in the crypt cells in the small intestine of chickens (Barfull et al., 2002) and rabbits (Hwang et al., 1991). Using quantitative PCR, Yang et al. (2011) showed that SGLT1 mRNA abundance was 30-35% lower in crypt cells compared to villus cells in the small intestine of neonatal pigs. The increased sensitivities of qPCR and the RNAscope assays may explain why SGLT1 mRNA was detectable in the crypt cells.

Using a glucose protectable phlorizin binding assay, Ferraris et al. (1992) measured the number of glucose transporters along the mouse intestinal villus-crypt axis. They reported high expression of glucose transporters from the villus tip to midvillus and low expression in the intestinal crypts. In response to a high carbohydrate diet, there was an increased number of transporters in the cells along the villus and in the crypts. Ferraris and Diamond (1992) further showed that the increase in the number of glucose transporters first appeared in the crypts. This suggests that the crypt cells, which are likely the stem cells, are capable of sensing the change in

glucose concentration and inducing the expression of glucose transporters before the enterocytes migrate up the villus.

At doh, there was a population of cells located just above the crypts that showed reduced staining for SGLT1 mRNA (indicated by red arrows in Fig. 6.2) compared with the more intense staining in the epithelial cells in the rest of the villus. This reduced staining pattern, which was most obvious in the duodenum, was similar to that observed for the peptide transporter PepT1 mRNA (Zhang and Wong, 2017). These weakly staining cells are likely progenitor transit amplifying cells, which are a population of cells that originate from stem cells in the crypt, but have not become a differentiated cell type (Carulli et al., 2014).

In conclusion, SGLT1 mRNA was expressed in the epithelial cells of the YS, with low expression between e11 to e17, peak expression at e19 and low expression at doh. In the small intestine, SGLT1 mRNA was expressed in cells along the intestinal villus-crypt axis. There was upregulation of SGLT1 mRNA in epithelial cells from e19 to doh, which was then maintained at a high level of expression from d 1 to d 7 posthatch. Cells in the crypt, which are presumably stem cells, also expressed SGLT1 mRNA, which showed that these cells can import glucose.

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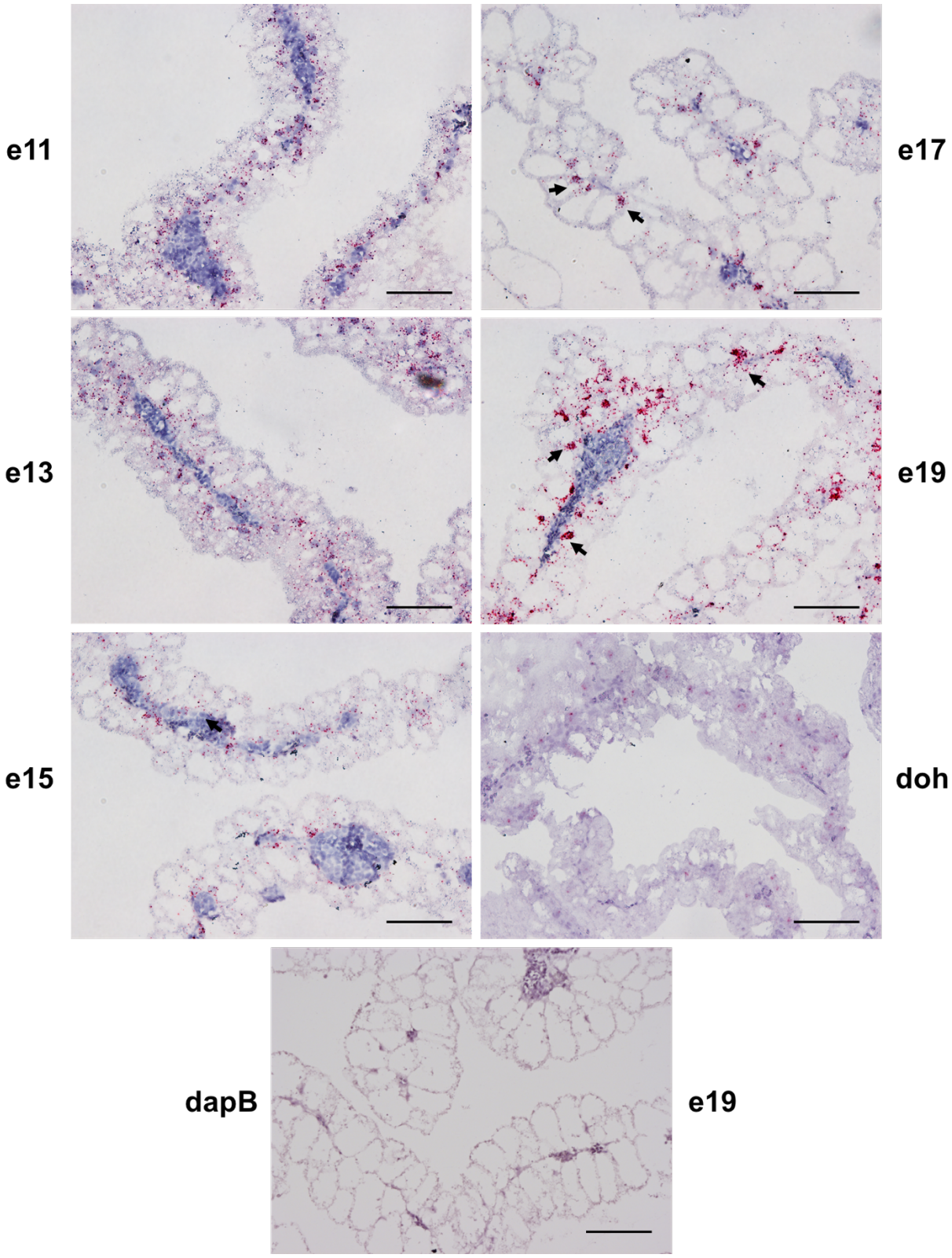
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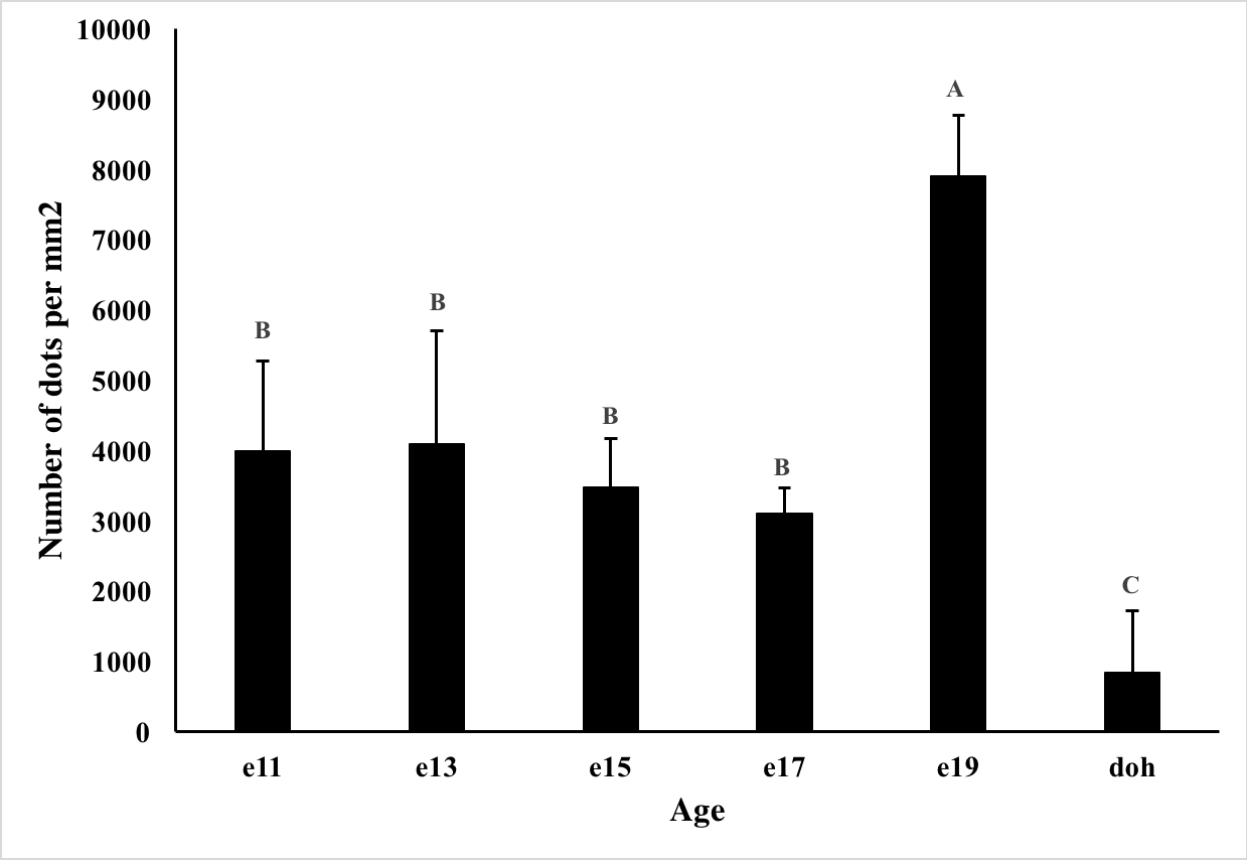

Figure 6.1 Expression of SGLT1 mRNA in chicken yolk sac from embryonic day 11 to day of hatch. A. Yolk sac samples were fixed in formalin, embedded in paraffin, and sectioned. SGLT1 mRNA was detected by in situ hybridization using the RNAscope 2.5 HD Detection kit (RED) at embryonic day 11 (e11), 13 (e13), 15 (e15), 17, (e17), 19 (e19) and day of hatch (doh) and is shown in the top six panels. The negative control dapB (bottom panel) was performed on yolk sac at e19, which was the age that showed the highest level of SGLT1 mRNA expression. The tissues were counterstained with hematoxylin. Images were captured using 200X magnification. The scale bars represent 0.1 mm. B. Average number of red dots (each dot represents a SGLT1 mRNA) per area (mm^2) of yolk sac. Data were obtained from images at

400X magnification and quantified using ImageJ software. Bars with different letters (A-C) are significantly different ($P < 0.05$).

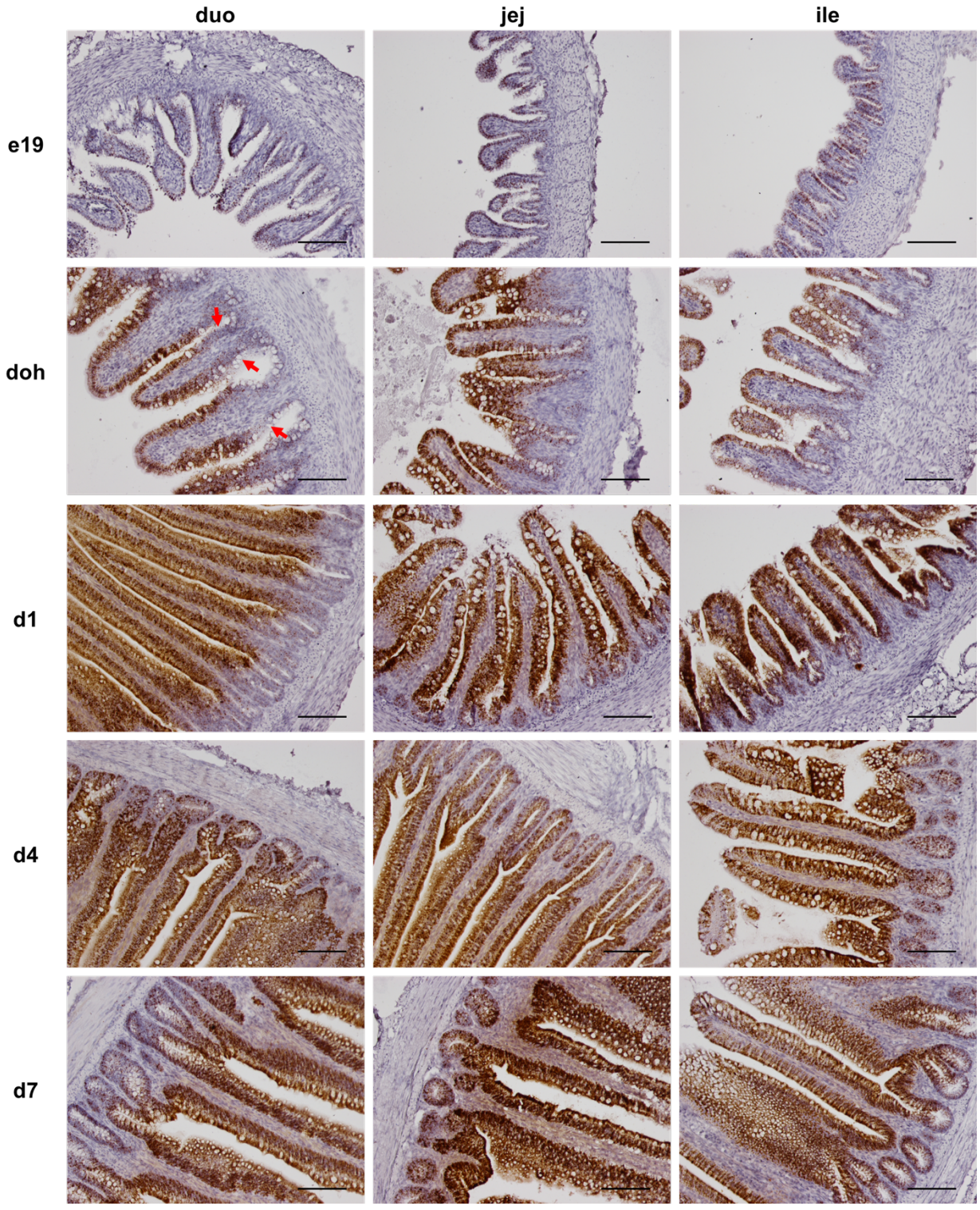

Figure 6.2 Expression of SGLT1 mRNA in duodenum (duo), jejunum (jej), and ileum (ile) from embryonic day 19 (e19) to d7 post-hatch. Intestinal segments were fixed in formalin, embedded

in paraffin, and sectioned. SGLT1 mRNA was detected by in situ hybridization using the RNAscope 2.5 HD Detection kit (BROWN). Each brown dot represents an SGLT1 mRNA molecule. The red arrows indicate the location of putative progenitor transit amplifying cells. Tissues were counterstained with hematoxylin. Images were captured using 200X magnification. The scale bars represent 0.1 mm.

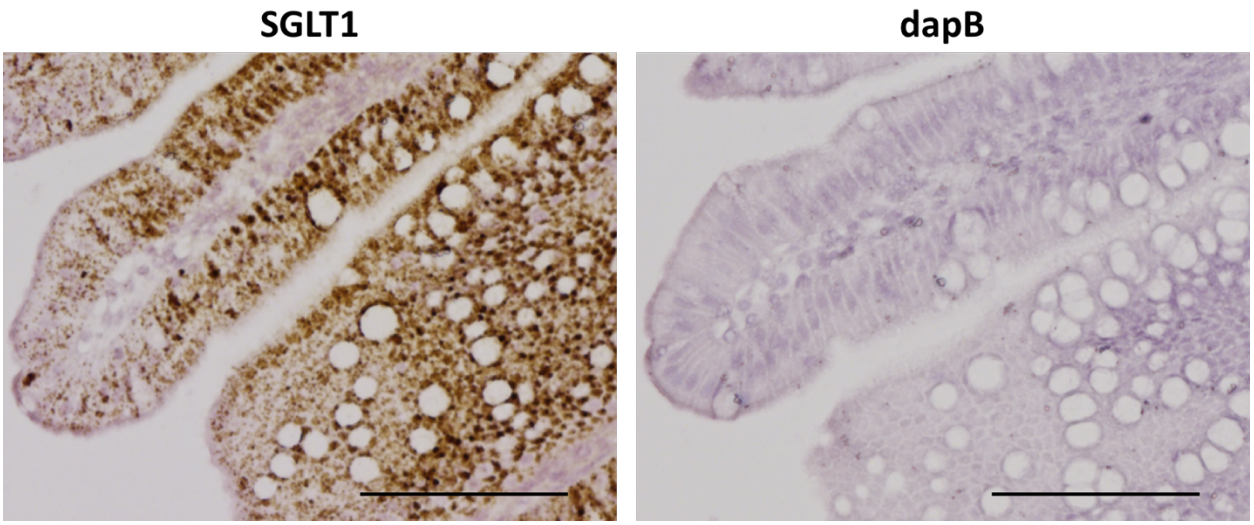

Figure 6.3 Expression of SGLT1 and dapB (negative control) mRNA in the jejunum at d4 posthatch. Serial sections from the jejunum were fixed in formalin, embedded in paraffin, and sectioned. SGLT1 and dapB mRNA were detected by in situ hybridization using the RNAscope 2.5 HD Detection kit (BROWN) on serial sections. Each brown dot represents an SGLT1 mRNA molecule. There was no staining with the dapB probe. Images were captured using 200X magnification. The scale bar represents 0.1 mm.

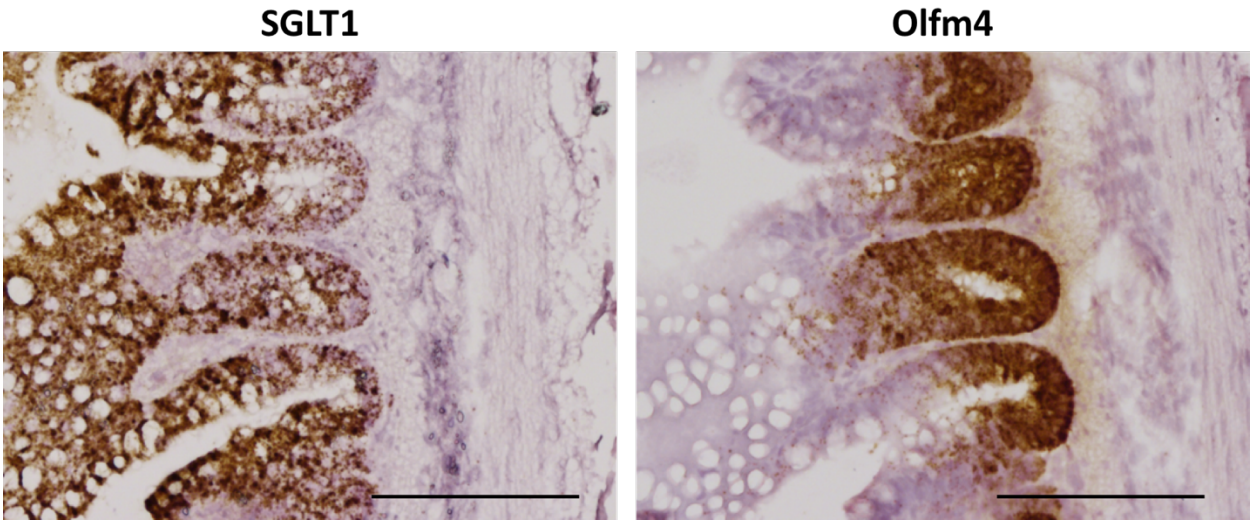

Figure 6.4 Expression of SGLT1 and Olfm4 mRNA in jejunum at d4 posthatch. Serial sections from the jejunum were fixed in formalin, embedded in paraffin, and sectioned. SGLT1 and Olfm4 mRNA were detected by in situ hybridization using the RNAscope 2.5 HD Detection kit (BROWN) on serial sections. Each brown dot represents an SGLT1 or Olfm4 mRNA molecule. Images were captured using 200X magnification. The scale bar represents 0.1 mm.

Chapter VII

Expression of avian β-defensin mRNA in the chicken yolk sac and small intestine

ABSTRACT

The chicken yolk sac (YS) and small intestine play important roles in nutrient absorption and immune function. They both serve as a physiological barrier to pathogens that may be present in the yolk or intestinal lumen during the embryonic and posthatch periods, respectively. Host defense peptides, which include the avian β defensins (AvBDs) are important members of the innate immune system. The objective of this study was to profile the mRNA expression pattern and distribution of AvBD mRNA in the chicken yolk sac and small intestine. Expression of AvBD1, 2, 7, and 10 mRNA showed development-specific expression in the YS, with low expression at embryonic day 7 (e7), which increased to e9 through e13 and then declined to e19. In situ hybridization analysis showed that in the YS AvBD10 mRNA was expressed in endodermal epithelial cells, while AvBD1, 2, and 7 mRNA were expressed in heterophils. In the small intestine, AvBD10 mRNA was expressed in the epithelial cells lining the villi at day of hatch, which decreased to a few cells above the intestinal crypts at d2 and d4 posthatch. AvBD1, 2 and 7 mRNA were expressed in heterophils that were localized in the connective tissue and lamina propria. The developmental expression and distribution of AvBD mRNA in the small intestine and YS suggest the importance of these genes to chicken immunity during the embryonic and early posthatch periods.

Key words: Avian β defensins, yolk sac, small intestine, qPCR, in situ hybridization.

INTRODUCTION

The yolk sac (YS) is a multifunctional organ that not only provides essential functions for the embryo while the embryonic organs are developing but also serves as a biological barrier and a first line of defense against pathogens that may be present in the yolk. The yolk sac is the first extra-embryonic membrane, which advances from the developing intestine and gradually encloses the yolk content during chicken embryogenesis (Patten, 1971; Mobbs and McMillan, 1979). It is not surprising that the yolk sac and small intestine share structural features. The yolk sac contains structures lined with endodermal epithelial cells (EEC) that project into the yolk, which serve to increase the surface area, much like the villi of the small intestine. A recent transcriptome analysis of the YS demonstrated that the YS possessed very similar biological functions in different vertebrate animals during the neonatal stage, emphasizing the immune and nutritional roles of the YS during the embryonic stage (Cindrova-Davies et al., 2017).

The YS plays a critical role in a number of biological functions such as hematopoiesis and nutrient uptake. The YS is the site for generating hematopoietic stem cells, which can either differentiate into precursors of red and white blood cells or spread via the blood circulation and colonize other embryonic organs (Sheng, 2010). Nutrients that are taken up from the yolk are transported by the extraembryonic blood circulation to the embryo (Noble and Cocchi, 1990). A variety of transporters for various nutrients such as amino acids, peptides, sugars, and lipids (Yadgary *et al.*, 2011, 2014; Speier *et al.*, 2012, Bauer et al., 2013) were found to be expressed in a development-specific manner in the YS. Using in situ hybridization, Zhang and Wong (2018) showed that the YS EEC expressed mRNA for the peptide transporter PepT1.

CHAPTER VII. AVBDS IN YOLK SAC AND INTESTINE

The developing chick is dependent upon maternally transmitted antibodies, which are deposited into the yolk, for protection against pathogens until it can synthesize its own antibodies (Kaspers et al., 1995; Kovacs-Nolan and Mine, 2012; Schijns et al., 2014). The YS plays an important role in passive immunity by taking up maternal yolk antibodies and transporting them intact into the embryonic circulation through a receptor-recognized, transcytotic process (Kowalczyk et al., 1985; Tesar et al., 2008).

Host defense peptides are part of the innate immune system and consist of a set of short (<100 amino acids) cationic polypeptides with broad spectrum antimicrobial activity. In avian species, the host defense peptides are classified as avian β-defensins (AvBDs), cathelicidins, and liver-expressed antimicrobial peptide 2 (LEAP2). In addition to the essential effects of β-defensins on enhancing host innate immunity against pathogens, defensins in humans have other important biological properties including the acceleration of wound repair by inducing the expression of mucin in the intestine and lung (Aarbiou et al., 2004; Otte et al., 2008), stimulation of the epithelial cell migration and proliferation (Aarbiou et al., 2002), and an anti-inflammatory effect (Kohlgraf et al., 2010). In chickens, the AvBD family consists of at least fourteen different members (AvBD1 to AvBD14), which show high sequence conservation (75% -95% identity) among avian species (Cuperus et al., 2013).

The expression of AvBD mRNA in chickens was found to be widely distributed among many different organs. AvBD1 and 2 were primarily isolated from chicken heterophils (Evans et al., 1994; Kannan et al., 2009), but also found to be expressed in intestinal epithelial cells of chicken embryos (Derache et al., 2009). Additionally, AvBD1 and 2 were highly expressed in many different tissues, such as bursa, lungs, testis and bone marrow (Lee et al., 2016; Lynn et al., 2004). The expression of AvBD4 was detected in chicken testis and was highly induced by

Salmonella infection (Anastasiadou et al., 2014). In the chicken gastro-intestinal tract, AvBD9 and 13 were predominantly expressed in the proventriculus. AvBD6, 8, 10 and 13 were moderately expressed in the small intestine in broiler chickens (Hong et al., 2012). The expression of AvBD was detectable in embryos early in development, with AvBD3, 5, 9, 10, 12, and 14 expressed in the chicken embryo from embryonic day 3 to 9 (Meade et al., 2009). At an early stage of posthatch, AvBD1 and 2 mRNA in the duodenum were high at day of hatch and declined during the first week posthatch (Bar-Shira and Friedman, 2006).

Because the embryo lacks the ability for acquired immunity, innate immunity, which is provided by the YS and the developing intestine, is critical for protecting the embryo. A number of studies have examined tissue-specific and development-specific changes in AvBD mRNA abundance in chickens using qPCR, which does not allow the identification of cells expressing AvBD mRNA. Thus, the objective of this research was to utilize in situ hybridization and qPCR to reveal the expression profiles and tissue distributions of selected AvBD in the YS during embryogenesis and the small intestine during the late embryonic and early post hatch stages.

MATERIAL AND METHODS

Animals and tissue collection

Fertile Cobb 500 eggs were obtained from a Cobb-Vantress hatchery (Wadesboro, NC) and incubated at 38°C with 60% relative humidity in a well-ventilated incubator (OVA-Easy Advanced, Brinsea, Titusville, FL). Embryonic chicks (n=6) were collected from eggs at embryonic day 7 (e7), e9, e11, e13, e15, e19, and day of hatch (doh) and killed by cervical dislocation. All animal procedures were approved by the Virginia Tech Institutional Animal Care and Use Committee. A piece of tissue (~1 sq cm) from the *area vasculosa* of the YS was

collected, rinsed with phosphate buffered saline (PBS) to remove residual yolk and cut into two pieces. One piece of tissue was frozen immediately in liquid nitrogen and stored at -80°C for RNA extraction. The other piece of tissue was fixed in neutral buffered formalin and embedded in paraffin for sectioning.

RNA extraction, cDNA synthesis and quantitative PCR

A 25 to 35 mg sample of yolk sac (n=6) was homogenized in TriReagent (Molecular Research Center Inc., Cincinnati, OH) using a tissue lyser. Total RNA was isolated following the manufacturer's instructions for the Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA). RNA concentration was quantitated using a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific, Pittsburgh, PA) and RNA quality was assessed using an Agilent 2100 Bioanalyzer (Santa Clara, CA). RNA with a RIN > 8.0 was used for quantitative PCR analysis.

Total RNA (1 mg) was reverse transcribed using random primers and the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Grand Island, NY). The qPCR reaction contained 5 µl Fast SYBR Green Mater Mix (Applied Biosystems), 1 µl each of forward and reverse primers (5 µM), 1.5 ul DEPC water, and 1.5 µl cDNA diluted 1:30. Each qPCR reaction was carried out in duplicate using an Applied Biosystems 7500 Fast Real-time PCR system (Thermo Fisher Scientific). The cycling conditions were: 95°C for 20 s, followed by 40 cycles of 90°C for 3 s and 60°C for 30 s. Primers for AvBD1, 2, 6, 7, 8, 10, 11, 12, 13 and LEAP2 were designed by Primer Express 3.0.1 (Applied Biosystems) and are listed in Table 1. Primers for β-actin and the ribosomal proteins (RPL4, RPLP0, and RPLP1) were tested as reference genes. GeNorm was used to identify the two most stable genes (Vandesompele et al., 2002). The geometric mean of RPLP0 and RPLP1 was used as a reference gene to calculate ΔCt. The

average ΔCt value of AvBD1 at e7 was used as the calibrator to calculate ΔΔCt and the fold change using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Differential blood cell staining

Formalin fixed paraffin embedded (FFPE) samples (n=3) were cut into 4-6 µm sections and mounted on Superfrost® Plus Microscope slides (Electron Microscopy Sciences, Hatfield, PA). The slides were incubated at 60°C, deparaffinized in xylene, rehydrated in a graded series of ethanol (100%, 90%, 70%, 50%), and rinsed twice in 1X PBS. Giemsa staining was performed according to the method described in Cuperus et al., (2016), with minor changes. Tissue sections were immersed into filtered 20% Giemsa's Azure Eosin Methylene Blue solution (Merck, Darmstadt, Germany) for 20 min and followed by 15 min in 2% acetic acid. The sections were dipped into 95% ethanol and transferred into acetone. After air drying, the tissues were covered by one drop of Clear Mount medium (American Master Tech Scientific, Inc, Lodi, CA) and visualized using a Nikon Eclipse 80i microscope and DS-Ri1 digital camera. After the slides were placed in xylene overnight, acidified ethanol was used to strip the Giemsa staining in order to perform in situ hybridization.

In situ hybridization analysis

The RNAscope and Basescope in situ hybridization (ISH) assays were carried out following the manufacturer's instructions (Advanced Cell Diagnostics, ACD, Newark, CA). The RNAscope probes for chicken AvBD2 (NM_001201399) and AvBD10 (NM_001001609.1) were custom designed to have 13 and 11 probe sets, respectively. Cells expressing AvBD2 and AvBD 10 mRNA were detected using the RNAscope 2.5 HD Detection Kit (BROWN). Because the

coding regions for AvBD1 (NM_204993) and AvBD 7 (NM_001001194) were shorter, only 3 probe sets for AvBD1 and 4 probe sets for AvBD7 could be designed. Cells expressing AvBD1 and AvBD7 mRNA were detected using the Basescope 2.5 HD Detection Kit (RED). Tissues were counterstained with 50% Gill # 2 hematoxlin (Sigma Aldrich, St Louis, MO) and then treated with 0.02% ammonia water. The slides were placed into xylene and air dried. One drop of Clear Mount medium (Thermo Fisher Scientific, Hampton, NH) was added and coverslips were gently mounted on top of the tissue. Images were captured and analyzed using a Nikon Eclipse 80i microscope and DS-Ri1 digital camera.

Statistical analysis

Gene expression data were analyzed by JMP Statistical Discovery Software v13.0 (SAS Institute, Cary, NC). Because the data were found to not follow a normal distribution, the data were analyzed using the Kruskal-Wallis nonparametric test. The Wilcoxon nonparametric test was used to compare samples to assess the significance of gene expression between different days. Significance was set at $P < 0.05$.

RESULTS

Gene expression pattern of AvBDs in chicken yolk sac

The mRNA abundance of 14 AvBD and liver expressed antimicrobial peptide 2 (LEAP2) were determined for the YS from e7 to e19 using qPCR. The expression profiles for nine AvBD are shown in Figure 7.1. Because AvBD3, 4, 5, 9 and 14 and LEAP2 mRNA showed very low expression, the data were not included in Figure 7.1. AvBD6, 8, 11, 12, and 13 mRNA showed low, constant expression at e7, e11, e15, and e19. In contrast, AvBD1, 2, 7 and 10 mRNA

showed high expression that was development-specific and were thus further analyzed at two additional time points (e9 and e13). AvBD1 mRNA increased from e7 to e13. AvBD2 mRNA increased from e7 to e9 and from e9 to e13 and then decreased from e13 to e19, while AvBD7 mRNA increased from e7 to e9 and then decreased from e13 to e15 and e19. AvBD10 mRNA showed the greatest expression, which increased approximately 10-fold from e7 to e9, maintained a high expression level from e9 to e13, and then declined to e15 and to e19.

Identification of cells expressing AvBD 1, 2, 7 and 10 mRNA in the YS by in situ hybridization and Giemsa staining

AvBD10 mRNA was predominantly localized in the epithelial cells of the yolk sac (Fig. 7.2). The intensity of AvBD10 mRNA staining increased from e7 to e11, declined from e11 to e15 and was barely detectable at e19. This pattern of AvBD10 mRNA expression was the same as that determined by qPCR (Fig. 7.1).

The distribution of cells expressing AvBD1, 2, and 7 mRNA in YS was totally different from that of cells expressing AvBD10. AvBD1 (red stain), AvBD2 (brown stain), and AvBD7 (red stain) mRNA were concentrated in cells located at the yolk sac blood islands and cells that were interspersed within yolk sac epithelial cells (Fig. 7.3).

To better identify cells that expressed AvBD1, 2, and 7 mRNA, sections were first stained with Giemsa to differentiate blood cell types and then processed for in situ hybridization. Cells expressing AvBD1, 2, and 7 mRNA in the YS blood islands, were acidophilic granulocytes, which were presumably heterophils (Fig. 7.4 and 7.5). Some of the acidophilic granulocytes (yellow arrows) in the YS expressed AvBD1, 2, and 7 mRNA, while other acidophilic granulocytes (green arrows) did not express AvBD1, 2, and 7 mRNA.

Identification of cells expressing AvBD 1, 2, 7 and 10 mRNA in the small intestine by in situ hybridization and Giemsa staining

Cells expressing AvBD10 mRNA in the small intestine from e20 to d2 are shown in Fig. 7.6. AvBD10 mRNA was predominantly found in intestinal epithelial cells at e20 and doh. By d2, the distribution of cells expressing AvBD10 mRNA was found to be limited to cells located above the intestinal crypt. By d4, only a few cells located above the intestinal crypts were found to express AvBD10 mRNA. Only a very few cells expressing AvBD1, 2, and 7 mRNA in the small intestine were identified, which were located in the lamina propria. Based on the Giemsa staining, these AvBD2 mRNA expressing cells were presumably heterophils (Fig. 7.7).

DISCUSSION

The chicken YS is an important organ that connects the yolk nutrients to the embryo and forms the initial barrier to prevent pathogens from encountering the embryo. The endodermal epithelia of the YS are the site for absorption and secretion of various nutrient transporters and digestive enzymes (Yadgary et al., 2011; Speier et al., 2012; Bauer et al., 2013; Zhang and Wong, 2017). Our in situ hybridization results showed that AvBD10 mRNA was highly expressed in YS epithelial cells, which suggests that the YS also plays a role in innate immunity by secreting host defense peptides. Meade et al (2009) examined AvBD mRNA expression in whole embryos at e6 and e9 and in head or abdomen of embryos at e12. They found that there was a 37-fold and 87-fold increase in AvBD10 mRNA at e6 and e9, respectively, relative to e3 and a 137-fold increase in AvBD10 mRNA at e12 in only the abdomen. These changes parallel our observed 10-fold increase in AvBD10 mRNA in the YS from e7 to e9 and e11.

High expression of AvBD10 mRNA in the YS raises the question of its functional role. One possibility is that AvBD10 mRNA, expressed in the YS during mid embryogenesis, may provide protection for the embryo in the event of a bacterial infection in the yolk. An alternative possibility is that AvBD10 may be serving a dual role that is unrelated to host immunity. Human alpha defensin has been found to be a critical factor associated with lipoprotein internalization by endothelial cell and smooth muscle cells by increasing the binding of lipoprotein to the vascular matrix (Bdeir et al., 1999; Higazi et al., 1997). During embryogenesis, the yolk provides lipid resources, which are absorbed in the form of lipoprotein (Noble and Cocchi, 1990). Transcriptome analysis of the YS during embryogenesis revealed high synthesis of lipoprotein from e13 in the YS (Bauer et al., 2013; Yadgary et al., 2014). Thus, the upregulation of AvBD10 mRNA in the YS may be associated with AvBD10-assisted lipoprotein absorption and resynthesis in the chicken YS.

The chicken YS also is the site for erythropoiesis and lymphopoiesis during embryogenesis similar to the mouse and human YS (Lassila et al., 1982; Nagai and Sheng, 2008; Palis and Yoder, 2001; Sheng, 2010). Cells expressing AvBD1, 2, and 7 mRNA in the YS were identified as acidophilic granulocytes or heterophils, which are the same as neutrophils in mammals. These results were consistent with the finding that AvBD1 and 2 have been isolated and enriched from chicken and turkey heterophils, (Evans et al., 1994; Harwig et al., 1994, Kannan et al., 2009). In addition, in our study not all heterophils expressed AvBD1, 2, and 7 mRNA. Some of the heterophils were apparently in an inactivated state and did not express AvBD mRNA. It is unknown if these heterophils express host defense peptides other than AvBD1, 2, and 7 or if these cells would express AvBD1, 2, and 7 mRNA at a later time, perhaps upon activation.

The small intestine is the major site of nutrient digestion and absorption during the posthatch stage and also plays an important role as an immune organ against enteric pathogens. Expression of HDP in the intestine has been profiled in chickens and turkeys. Hong et al (2012) showed that there was high expression in the jejunum of AvBD8, 10 and 13 mRNA and low expression of AvBD1, 6, 9, 11 and 12 mRNA in 3-wk old Cobb chickens; while there was high expression of AvBD8 and 13 mRNA and low expression of AvBD1, 6, 9, 10, 11, and 12 mRNA in 3-wk old Ross chickens. Bar-Shira and Friedman (2006) showed that AvBD1 and 2 mRNA decreased from doh to d7 and then increased from d7 to d14 in the duodenum of Ross chickens. Using immunohistochemistry, Cuperus et al. (2016) identified cells in both the villi and crypts that expressed AvBD9. AvBD9 expressing cells, which were identified as enteroendocrine cells, were rare at e14 and increased with developmental age. Similarly, Terada et al. (2018) used immunohistochemistry to localize AvBD2-positive cells to the lamina propria beneath epithelial cells of the villi and crypts in both the ileum and cecum. In the cecum, the frequency of AvBD2-positive cells increased from e19 to doh and then decreased on d7. More limited studies on AvBD expression have been conducted in turkeys. Hamad et al., (2017) showed that AvBD1, 2, 8, 9, and 13 mRNA expression increased from doh to d28 posthatch, while AvBD10 mRNA decreased from doh to d28 posthatch.

In this study, we found that AvBD10 mRNA showed a similar decrease after hatch. AvBD10 mRNA was expressed in enterocytes all along the villus at e20 and doh, which became limited to a population of enterocytes above the intestinal crypts at d2 and expressed in only a few enterocytes above the crypts at d4. During this time, expression of AvBD10 mRNA in enterocytes was low compared to the strong expression of the peptide transporter PepT1 mRNA (Zhang and Wong, 2017). Su et al (2017) showed that there was strong expression of LEAP2

mRNA in the enterocytes of d28 Ross chickens, but did not examine developmental changes. These results suggest that at hatch, enterocytes express mRNA for not only nutrient transporters such as PepT1 but also HDP such as AvBD10, but as the intestine matures, the expression of some HDP genes such as AvBD10 decreases.

In the intestine similar to the YS, there were heterophils that expressed AvBD1, 2, and 7 mRNA and likely originated in the bone marrow. Lee et al. (2016) showed that AvBD1 to AvBD7 expression was predominantly in the chicken bone marrow. This pattern of cell staining for AvBD1, 2 and 7 mRNA is similar to the foci of granulocytes containing heterophils located in the lamina propria and connective tissue of the small intestine in newly hatched chicks (Bar-Shira and Friedman., 2006).

In summary, the chicken yolk sac expresses a number of AvBD mRNA. Only AvBD1, 2, 7 and 10 showed development-specific expression with an increase from e7 to e13 and a decrease from e15 to e19. In situ hybridization showed that AvBD10 mRNA was expressed greatest in the enterocytes of the YS, with peak expression around e9 to e13. This adds a potential role in innate immunity to the repertoire of the epithelial cells of the YS. In the intestine, AvBD10 was expressed in the enterocytes along the villus at hatch, which decreased to only a few AvBD10 expressing cells by d4 posthatch. Granulocytes, which are presumably heterophils, expressed AvBD1, 2 and 7 mRNA and were present in both the YS and the small intestine.

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Figure Captions

Figure 7.1 Relative expression of AvBD mRNA in the yolk sac from embryonic day 7 to 19. The fold change for AvBD expression at embryonic day 7 (e7), 9 (e9), 11 (e11), 13 (e13), 15 (e15) and 19 (e19) was calculated using the $2^{-\Delta\Delta Ct}$ method. AvBD1 at e7 was used as the calibrator. Bars with different letters indicate a significant difference within a gene ($P< 0.05$).

Figure 7.2 Distribution of AvBD10 mRNA in the yolk sac from embryonic day 7 to 19. Sections were obtained from formalin-fixed paraffin embedded yolk sac samples at embryonic day 7 (e7), 11 (e11), 15 (e15), and 19 (e19). In situ hybridization for AvBD10 mRNA was performed according to the RNAscope method. The brown staining indicates AvBD10 mRNA, which was localized to the yolk sac endodermal epithelial cells. The sections were counterstained with hematoxylin. The scale bar represents 0.1 mm.

Figure 7.3 Distribution of AvBD1, 2, and 7 mRNA in the yolk sac from embryonic day 7 to 19. Sections were obtained from formalin-fixed paraffin embedded yolk sac samples at embryonic day 7 (e7), 11 (e11), 15 (e15), and 19 (e19). In situ hybridization for AvBD1 and 7 mRNA used the Basescope method with red staining, while AvBD2 mRNA used the RNAscope method with brown staining. The sections were counterstained by hematoxylin. The scale bar represents 0.1 mm.

Figure 7.4 Giemsa staining and RNAscope in situ hybridization for AvBD2 mRNA in the chicken yolk sac. Sections were obtained from formalin-fixed paraffin embedded yolk sac at embryonic day 15. Yolk sac sections were sequentially stained with Giemsa's azure eosin

methylene blue (A, B and C) and then processed for in situ hybridization using the RNAscope method (D, E, and F). Presence of AvBD2 mRNA was revealed by brown staining. The green arrows indicate heterophils that did not express AvBD2 mRNA, while the yellow arrows indicate heterophils that did express AvBD2 mRNA. The scale bar represents 0.05 mm.

Figure 7.5 Giemsa staining and RNAscope in situ hybridization for AvBD1 and AvBD7 mRNA in the chicken yolk sac. Sections were obtained from formalin-fixed paraffin embedded yolk sac at embryonic day 15. Yolk sac sections were sequentially stained with Giemsa's azure eosin methylene blue (A and B) and then processed for in situ hybridization using the Basescope method (C and D). Presence of AvBD1 and 7 mRNA was revealed by red staining. The green arrows indicate heterophils that did not express AvBD2 mRNA, while the yellow arrows indicate heterophils that did express AvBD2 mRNA. The scale bar represents 0.1 mm.

Figure 7.6 Expression of AvBD10 mRNA in the small intestine from embryonic day 20 to day 4 posthatch. Sections were obtained from formalin-fixed paraffin embedded duodenum (duo), jejunum (jej), and ileum (ile) at embryonic day 20 (e20), day of hatch (doh) and days 2 (d2) and 4 (d4) posthatch. AvBD10 mRNA was detected using the RNAscope in situ hybridization method and revealed as brown staining. The sections were counterstained with hematoxylin. The scale bar represents 0.05 mm.

Figure 7.7 Distribution of AvBD1, 2 and 7 mRNA in the duodenum at embryonic day 20. Sections were obtained from formalin-fixed paraffin embedded duodenal tissue at embryonic day 20 and analyzed for the presence of AvBD1 (a), AvBD2 (b) and AvBD7 (c) mRNA. In situ

hybridization for AvBD1 and AvBD7 mRNA were performed using Basescope and AvBD2 mRNA was performed using RNAscope. To further identify cells expressing AvBD2 mRNA, sections were first stained with Giemsa (d) and then processed for AvBD2 mRNA by RNAscope (e). The red staining granulocytes, which were identified as chicken heterophils, expressed AvBD2 mRNA. The scale bars represent 0.1 mm.

Table 7.1. Forward and reverse primers for quantitative PCR.

Gene	Description[1]	Forward primer (5'→3')	Reverse primer (5'→3')	Amplicon size (bp)	Accession no.
AvBD1		GAGTGGCTTCTGTGCATTTCTG	TTGAGCATTTCCCACTGATGAG	62	NM_204993.1
AvBD2		GCGGGACATGCTGTTCTGT	GCTGGGACACCCTCCAAAG	56	NM_204992
AvBD6		GCCCTACTTTTCCAGCCCTATT	GGCCCAGGAATGCAGACA	63	NM_001001193
AvBD7		GGCCATGAGGATCCTTTACCT	AACACCCTGGAGCACCACAA	58	NM_001001194.1
AvBD8		ATGCGCGTACCTAACAACGA	TGCCCAAAGGCTCTGGTATG	95	NM_001001781.1
AvBD10		CAGACCCACTTTTCCCTGACA	CCCAGCACGGCAGAAATT	64	NM_001001609.2
AvBD11		GGTACTGCATCCGTTCCAAAG	GCATGTTCCAAATGCAGCAA	56	NM_001001779.1
AvBD12		TGTAACCACGACAGGGGATTG	GGGAGTTGGTGACAGAGGTTT	114	NM_001001607.2
AvBD13		CAGCTGTGCAGGAACAACCA	CAGCTCTCCATGTGGAAGCA	59	NM_001001780.1
β-actin		GTCCACCGCAAATGCTTCTAA	TGCGCATTTATGGGTTTTGTT	78	NM_205518.1
cRPL4		TCAAGGCGCCCATTCG	TGCGCAGGTTGGTGTGAA	55	NM_001007479.1
cRPLP0		GCGATTGCTCCCTGTGATG	TCTCAGGTCCGAGACCAGTGT	59	NM_204987.2
cRPLP1		TCTCCACGACGACGAAGTCA	CCGCCGCCTTGATGAG	63	NM_205322.1

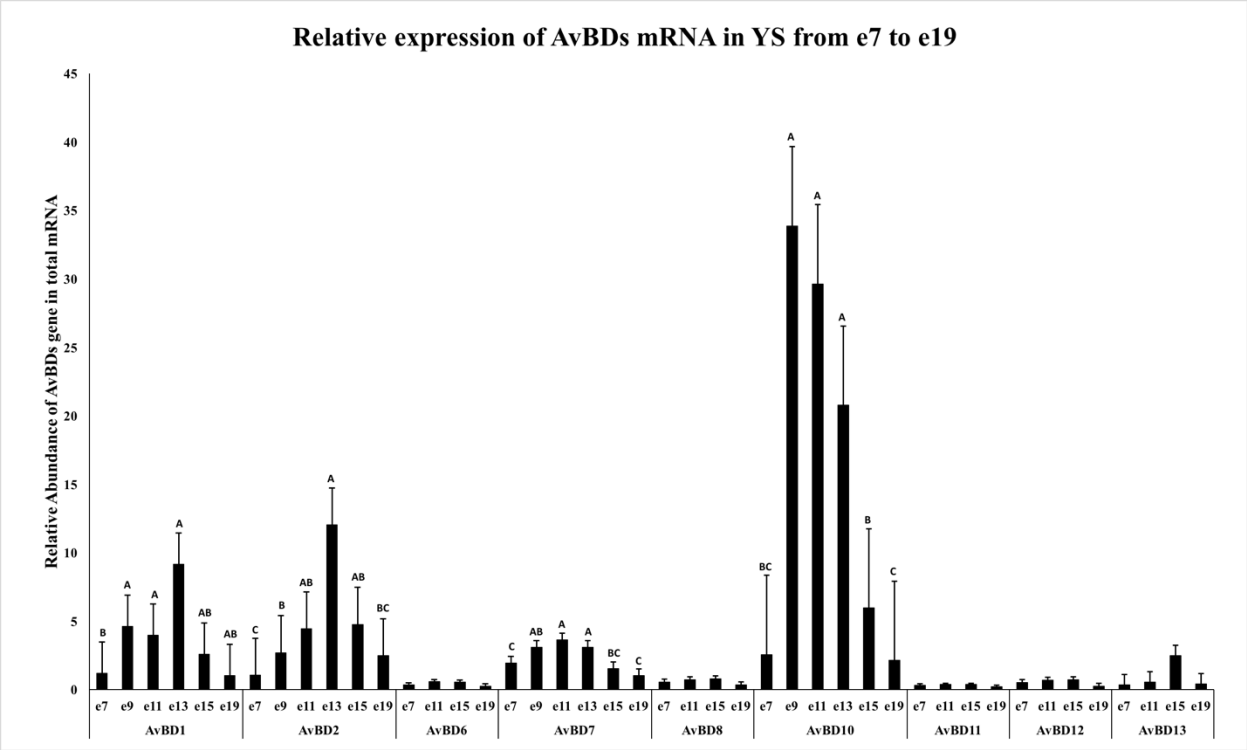
Relative expression of AvBDs mRNA in YS from e7 to e19
Relative Abundance of AvBDs gene in total mRNA
45
40
35
30
25
20
15
10
5
0
e7 e9 e11 e13 e15 e19
AvBD1
AvBD2
AvBD6
AvBD7
AvBD8
AvBD10
AvBD11
AvBD12
AvBD13

Figure 7.1

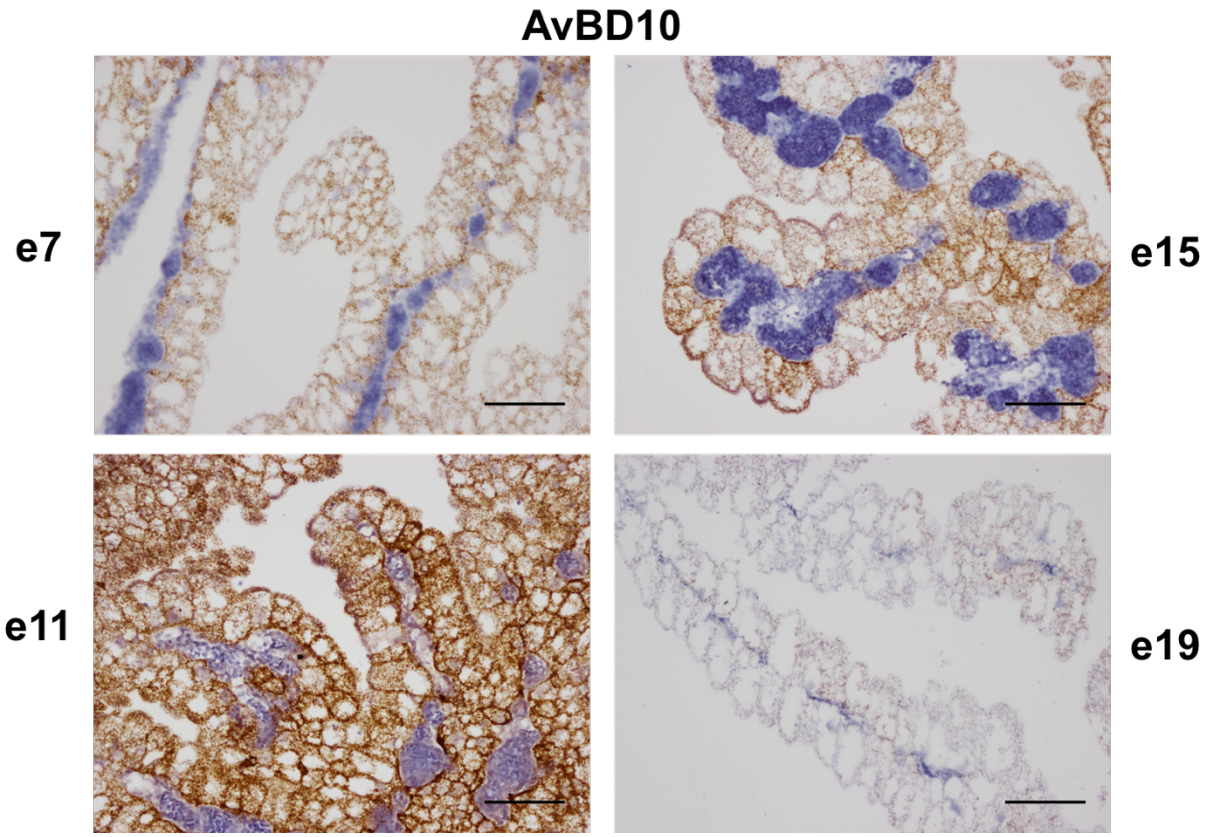

Figure 7.2

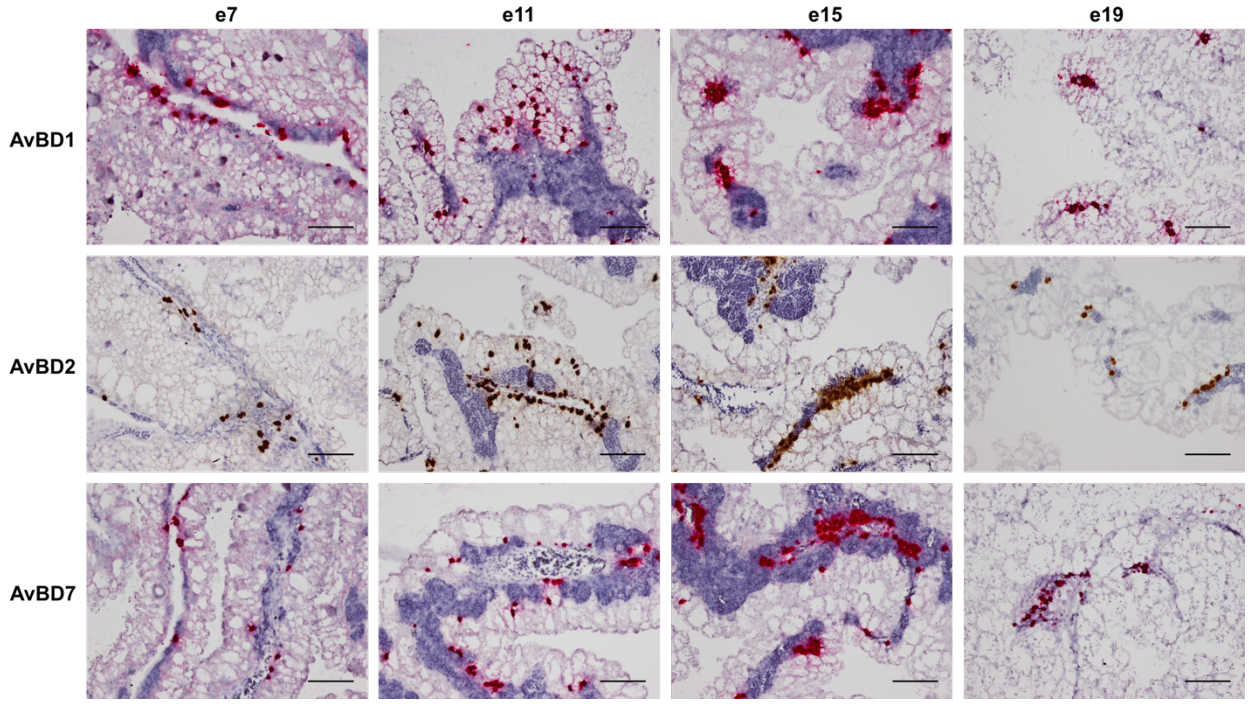

Figure 7.3

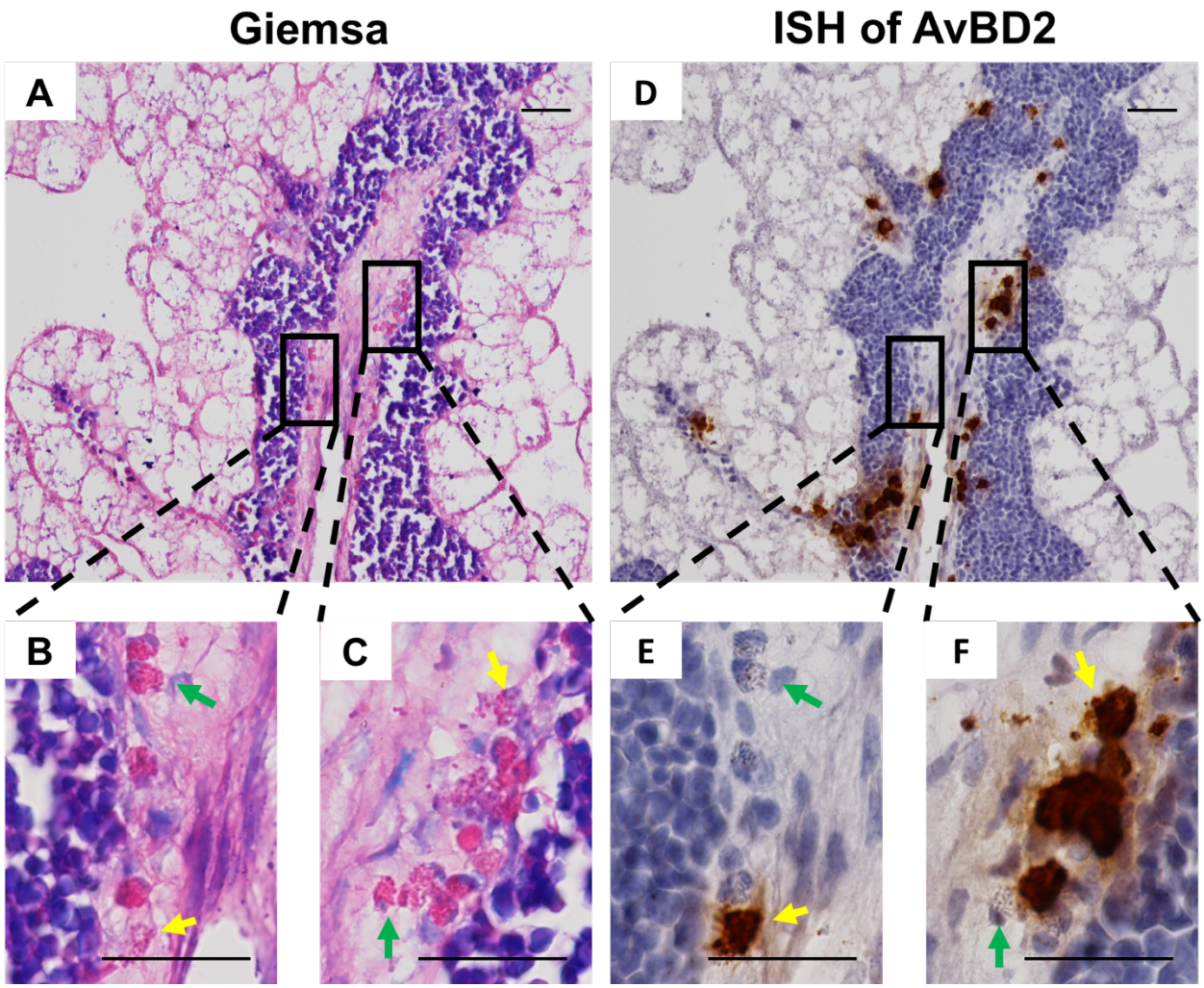

Figure 7.4

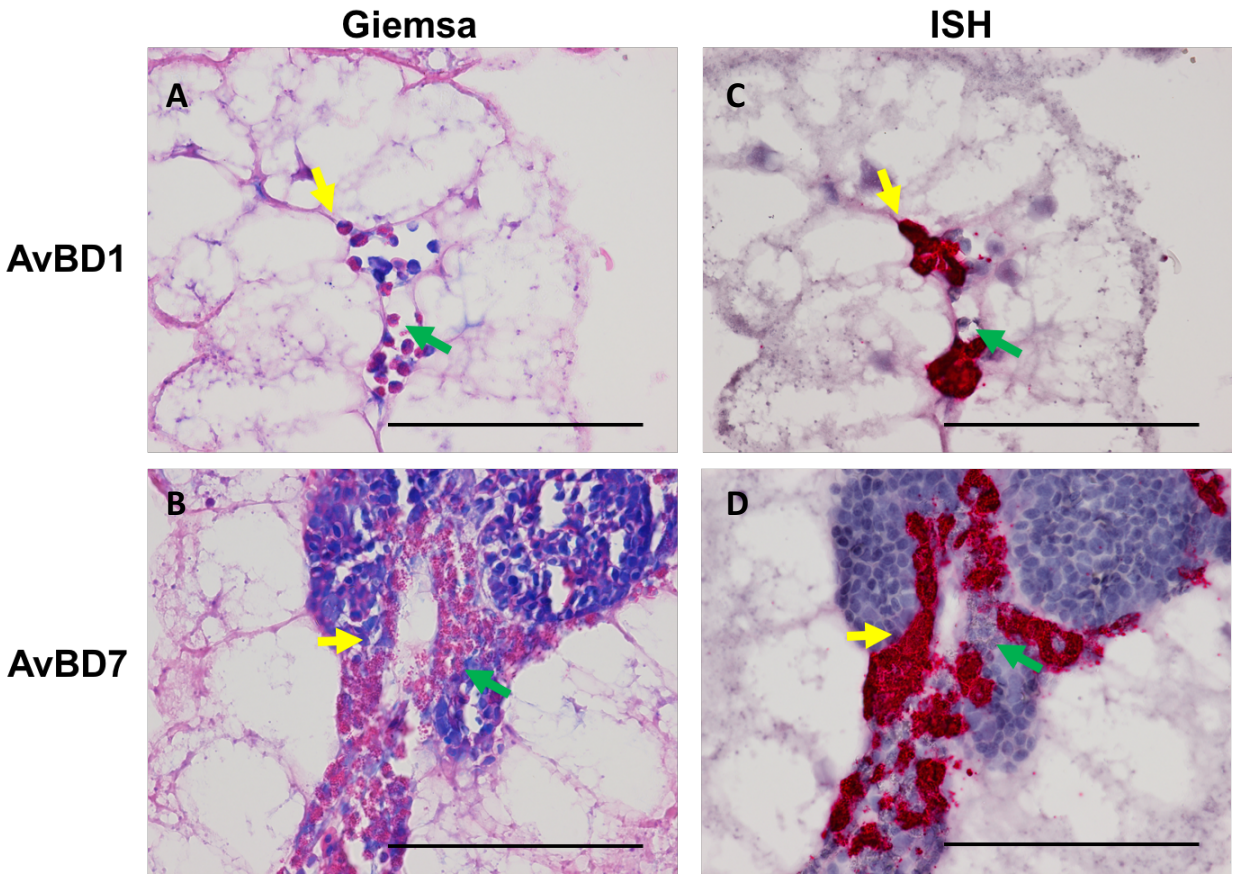

Figure 7.5

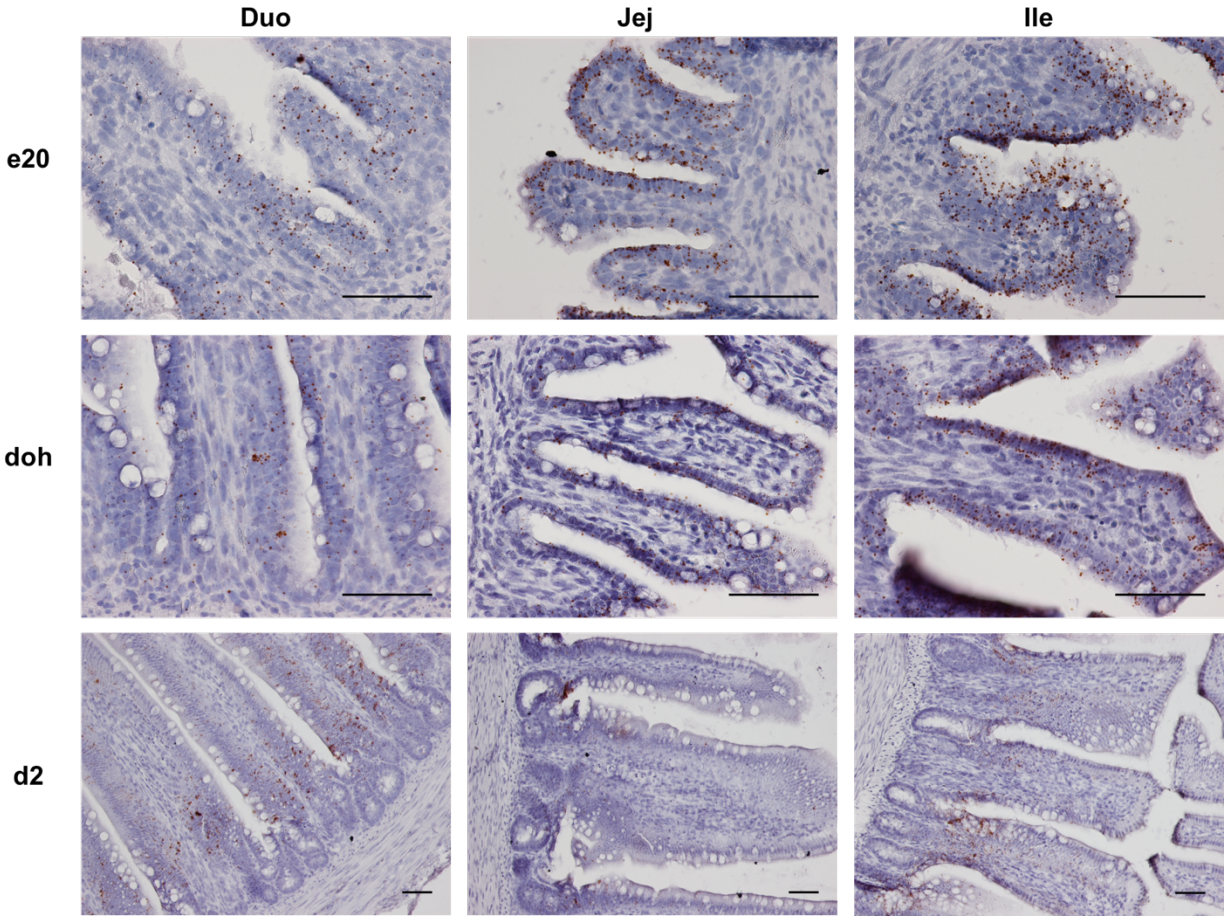

Figure 7.6

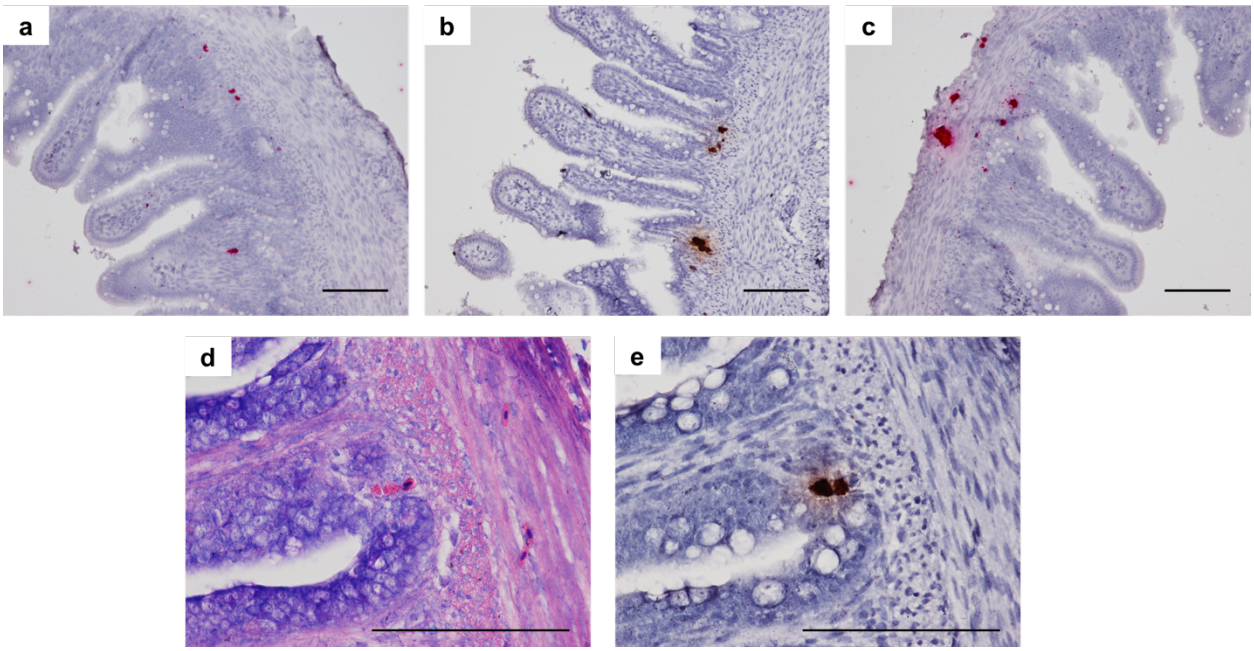

Figure 7.7

Chapter VIII

Immunofluorescence of PepT1 and Olfm4 and in situ hybridization of LEAP2 and EAAT3 in the chicken yolk sac and small intestine

BACKGROUND

Oligopeptides are absorbed by PepT1-mediated transport in the small intestine (Spanier, 2014). In humans and rats, the distribution of PepT1 protein was at the brush border membrane of the enterocyte (Ziegler et al., 2002; Oglihara et al., 1999). However, the location of PepT1 protein expressed in chickens is still unknown.

Olfm4 is a robust intestinal stem cell marker, which expresses high levels of both protein and mRNA in the intestinal crypt (Schuijer et al., 2014). Olfm4 protein could function like mucin to coat the intestinal mucosal layer because it could form large polymers through disulfide connections (Gersemann et al., 2012). The function of Olfm4 protein in the chicken small intestine is still unknown.

EAAT3 (Excitatory Amino Acid Transporter 3) is an anionic amino acid transporter that is responsible for the uptake of glutamate in the small intestine. EAAT3 has been found to play a critical role in the proliferation of intestinal epithelial cells in pigs and growth of the embryonic intestine in chickens (Li et al., 2014; 2016). LEAP2 (Liver Expressed Antimicrobial Peptide 2) is one of the important host defense peptides for chicken innate immunity. A previous study in our lab showed that EAAT3 and LEAP2 mRNA were expressed by enterocytes in the chicken small intestine. However, the location of cells expressing EAAT3 and LEAP2 mRNA in the yolk sac is still unknown.

MATERIAL AND METHODS

Animals

The broiler chickens used in this study are the same as those described in Chapter IV. The intestinal and yolk sac samples were also processed to make paraffin blocks for in situ hybridization as described in Chapter IV. Frozen blocks were made by embedding yolk sac and intestinal samples in the OCT (Optimum Cutting Temperature) gel and freezing in liquid nitrogen.

Immunofluorescence assay

The immunofluorescence (IF) assay is used for localization of the target protein in tissues or cells. In the IF assay in this study, a rabbit anti-PepT1 polyclonal antibody was generated by EZBiolab (Carmel, IN). The sequence of the peptide used for stimulating a host immune reaction was CQIKQDPDLHGKESEA. The Olfm4 antibody was produced by GenScript (Piscataway, NJ). The best strategy for combining RNAscope fluorescence in situ hybridization (FISH) and IF was to first perform FISH and then apply IF on the same tissue section. The Olfm4 RNAscope FISH was conducted as illustrated in Chapter IV. The red dye was used because it was also fluorescent. The secondary antibody (goat anti-rabbit, Dylight- 488) that bound primary antibodies was purchased from Vector Laboratories (Burlingame, CA). The OCT frozen slides were prepared for the IF assay.

Pretreatment steps: Frozen slides that were stored at -80 °C were pre-warmed at room temperature for 30 minutes. The slides were rinsed in 1X PBS for three times. Afterwards, 1% H_2O_2 was placed on the slides to quench the activity of peroxidase in the tissue. A 1mM sodium

citrate solution (pH = 6.0) was heated to 98 °C to 100°C for antigen unmasking. The slides were put into the boiling sodium citrate solution for 15 minutes and then washed in 1X PBS.

Immuno-signal detection steps: In order to diminish the background noise from nonspecific binding, the slides were first incubated with 5% goat serum blocking solution at 40°C for 2 hours. The slides were then incubated at 40°C for 2 hours with the primary antibody (rabbit anti-chicken PepT1 or rabbit anti-chicken Olfm4), which was diluted (1:200) with blocking solution. Afterwards, the slides were rinsed with 1X PBS and then incubated at 40°C for 1 hour with the secondary antibody (goat anti-rabbit IgG) which was diluted (1:200) with blocking solution. Finally, the slides were washed with 1X PBS and incubated for 2 minutes with DAPI which was diluted (1:1000) with blocking solution. Clear Mount solution was dropped on the slides and the coverslips were gently placed to cover the tissues. The slides were observed and analyzed using a Nikon Eclipse 80i microscope and DS-Ri1 digital camera.

RESULTS

The immunofluorescence assay was performed to detect the distribution of PepT1 protein in the chicken yolk sac and small intestine. In the yolk sac, samples from e15 and e19 were tested. The results are displayed in Figure 8.1. On both e15 and e19, PepT1 protein was distributed at the basolateral membrane of yolk sac endodermal epithelial cells. There were also some PepT1 protein localized at the brush border membrane of the endodermal epithelial cells, but the fluorescence intensity was not as strong as that on the basolateral membrane.

In the chicken small intestine, the distribution of PepT1 protein was identified in both the embryonic and post-hatch stages. The results are shown in Figure 8.2. PepT1 protein was

intensively localized at the brush border membrane of intestinal epithelial cells along the villus both in the pre-hatch and post-hatch periods.

In order to better understand the mRNA and protein distribution of Olfm4, FISH and IF were performed on the same intestinal samples. The results (Figure 8.3) indicated that both Olfm4 protein and mRNA were located in the intestinal crypts. Additionally, Olfm4 mRNA was primarily distributed in the cytoplasm of intestinal crypt cells. Olfm4 protein was located in the crypt cells and also found to be secreted only to the intestinal lumen side.

The distribution of EAAT3 and LEAP2 mRNA in the chicken yolk sac from e11 to e19 is shown in Figure 8.4 and Figure 8.5, respectively. The expression of EAAT3 mRNA was widely distributed in the yolk sac endodermal epithelial cells from e11 to e19. However, there were no differences in EAAT3 mRNA expression between embryonic days. The expression of LEAP2 mRNA was barely detectable in the yolk sac from e11 to e15, but localized in cells in the yolk sac blood islands at e17 and e19. The expression of LEAP2 mRNA in the small intestine at an early stage (day of hatch) is shown in Figure 8.6. LEAP2 mRNA was expressed by enterocytes mainly from the mid to the tip of the villus in the small intestine.

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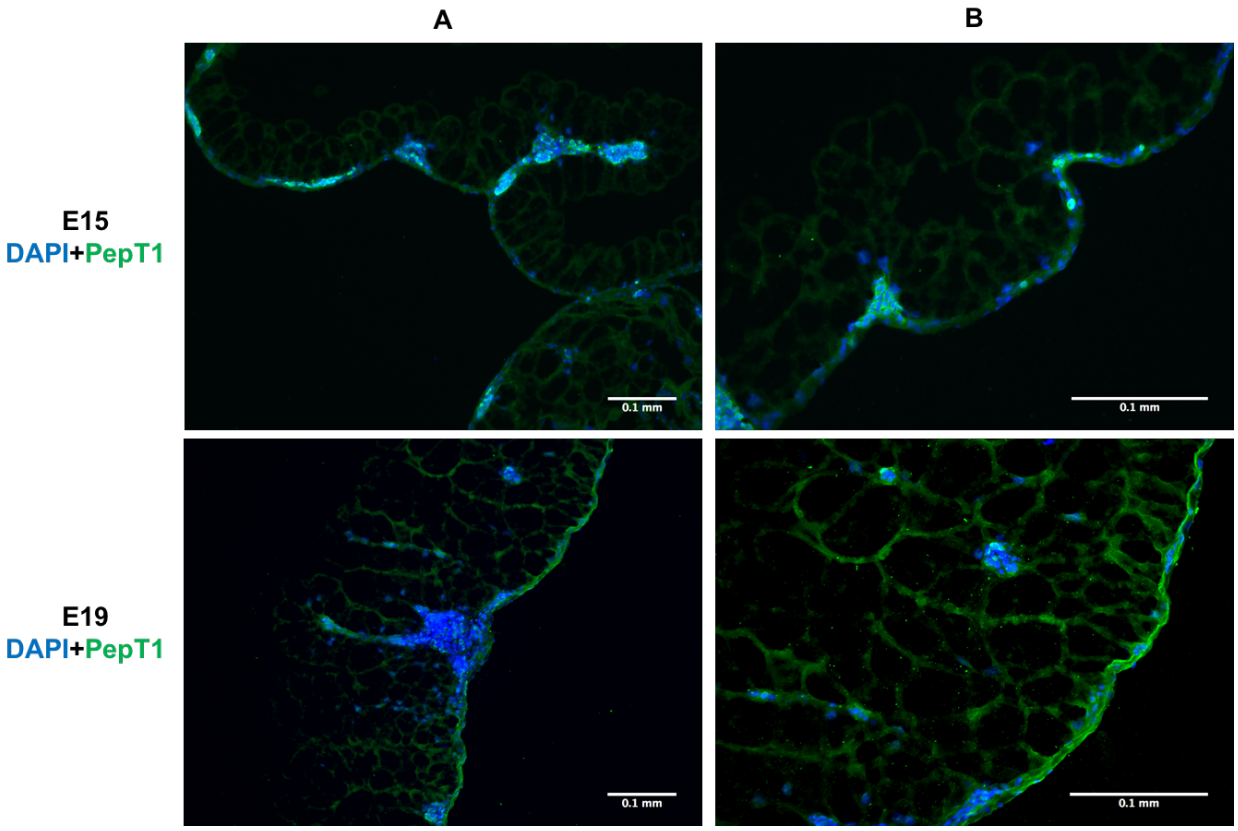

Figure 8.1 Distribution of PepT1 protein in the chicken yolk sac at E15 and E19. Intestinal samples were fixed in formalin, embedded and frozen in OCT. Expression of PepT1 protein in cells was tested by immunofluorescence assay. The green fluorescence indicates the location of PepT1 protein. The cell nuclei were stained with DAPI. Images were captured at 100x (A) and 200x (B) magnification. The scale bar represents 0.1 mm.

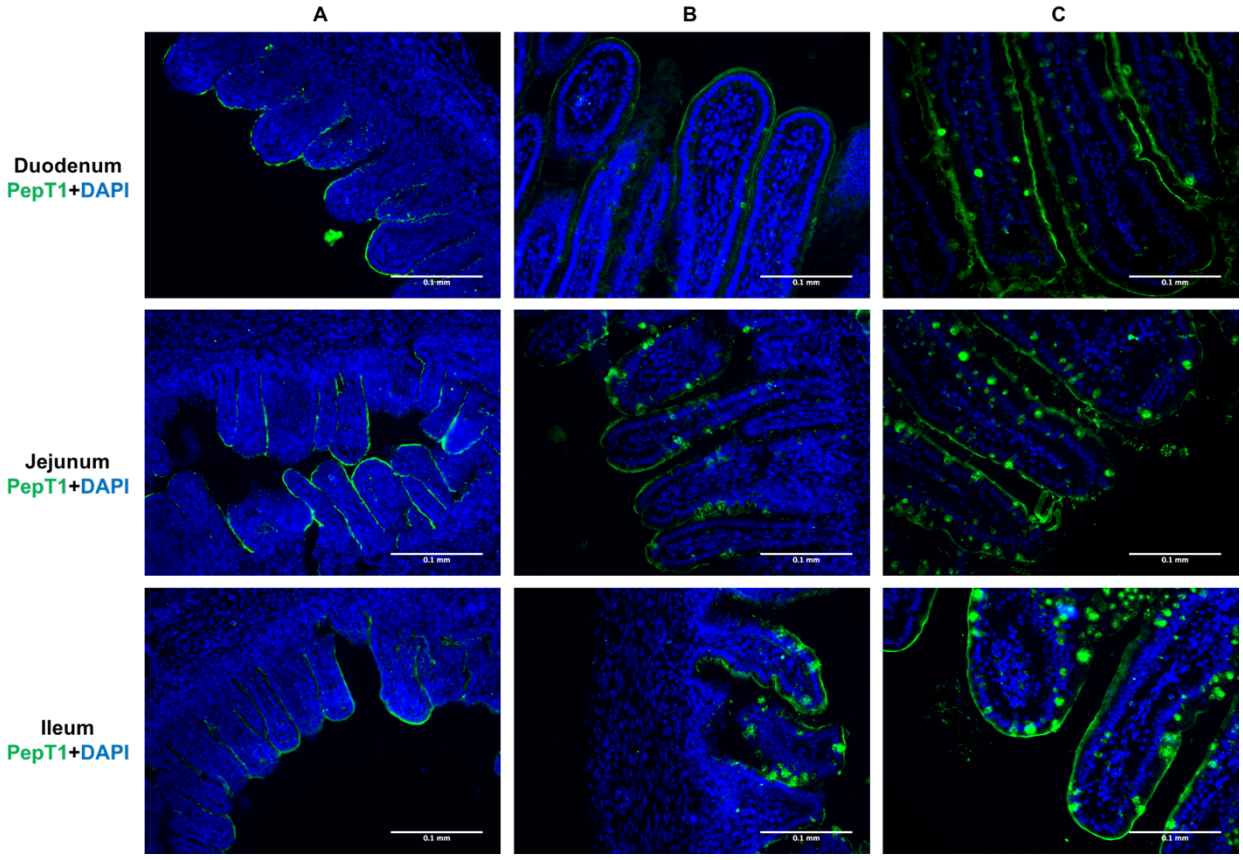

Figure 8.2 Distribution of PepT1 protein in duodenum, jejunum and ileum at e19 (A), day of hatch (B), and day 2 posthatch (C). Intestinal samples were fixed in formalin, embedded and frozen in OCT. Expression of PepT1 protein in cells was tested by an immunofluorescence assay. The green fluorescence indicates the location of PepT1 protein. The cell nuclei were stained with DAPI. Images were captured using 200x magnification. The scale bar represents 0.1 mm.

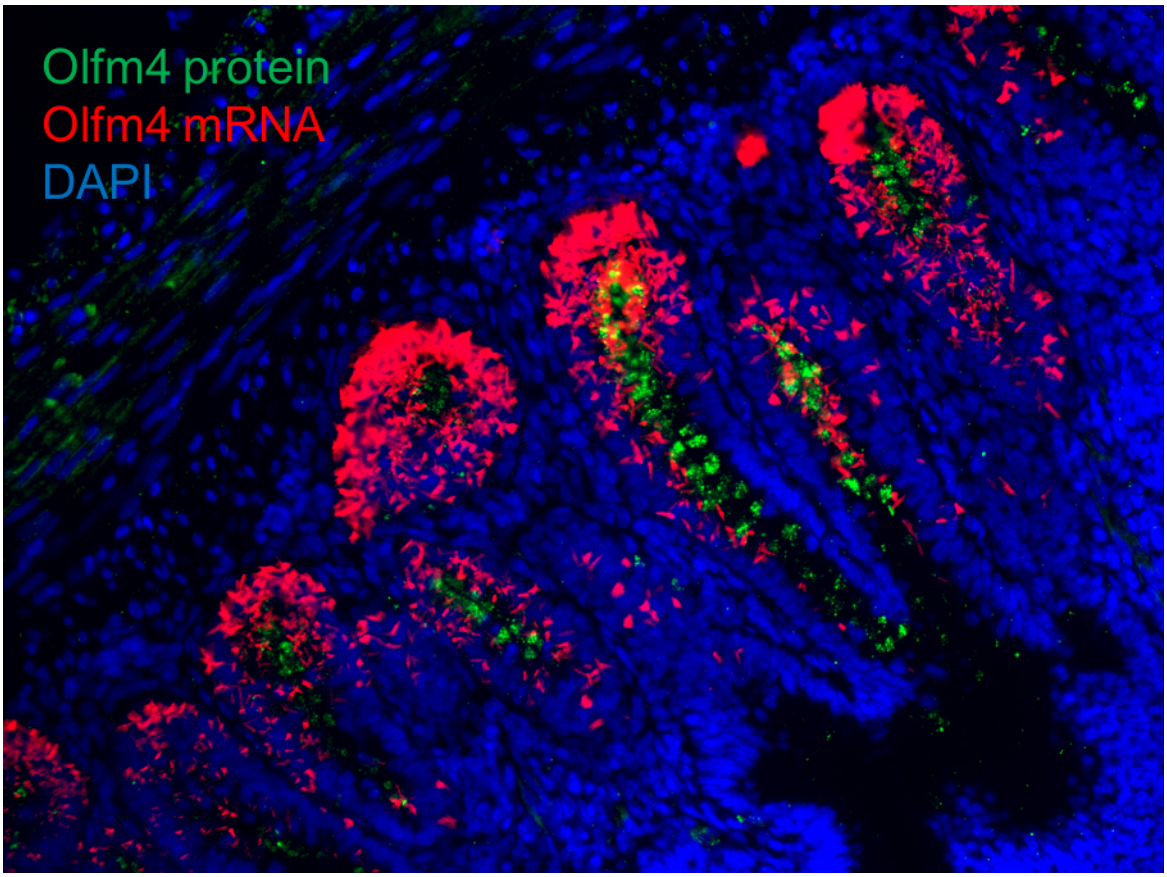

Figure 8.3 Co-expression of Olfm4 protein (Green) and mRNA (Red) in the chicken small intestine posthatch (D4). Intestinal samples were fixed in formalin, embedded and frozen in OCT. Expression of PepT1 protein in cells was tested by an immunofluorescence assay. The cell nuclei were stained with DAPI. Images were captured using 200x magnification.

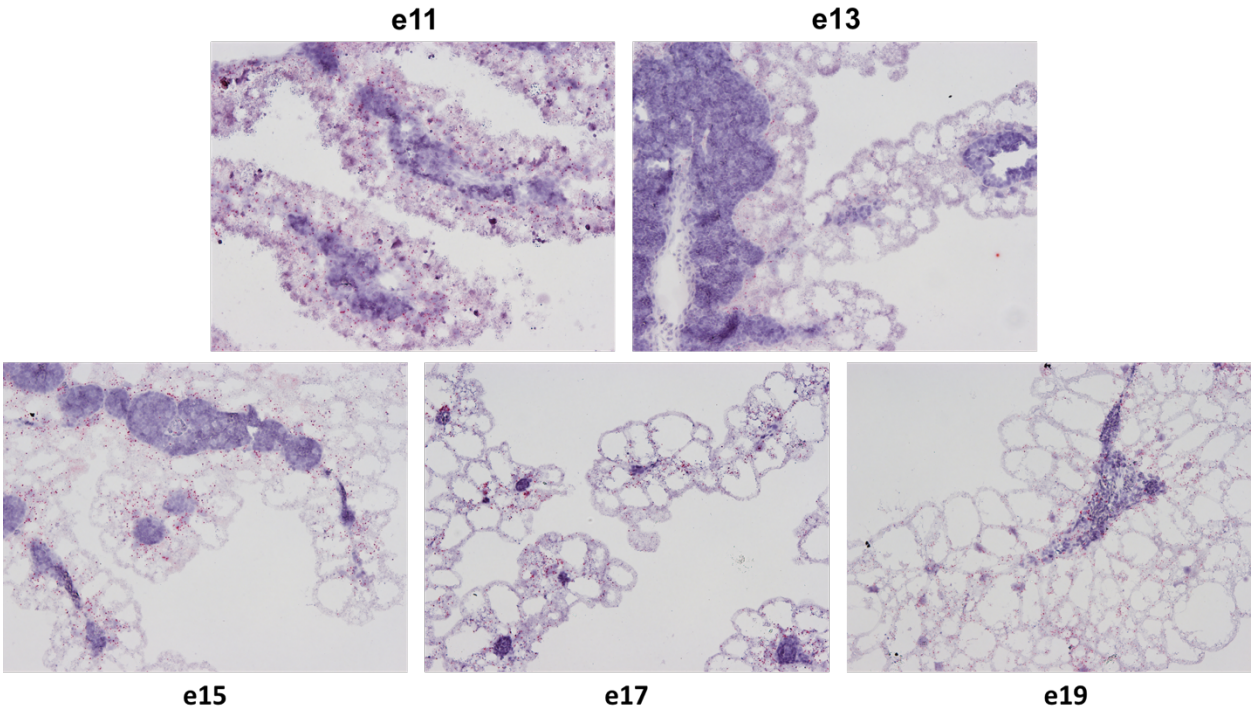

Figure 8.4 In situ hybridization of EAAT3 mRNA in the chicken yolk sac from e11 to e19. The red dots in each picture represent EAAT3 mRNA. The yolk sac samples were counterstained with 50% hematoxylin. All images were captured using 200x magnification.

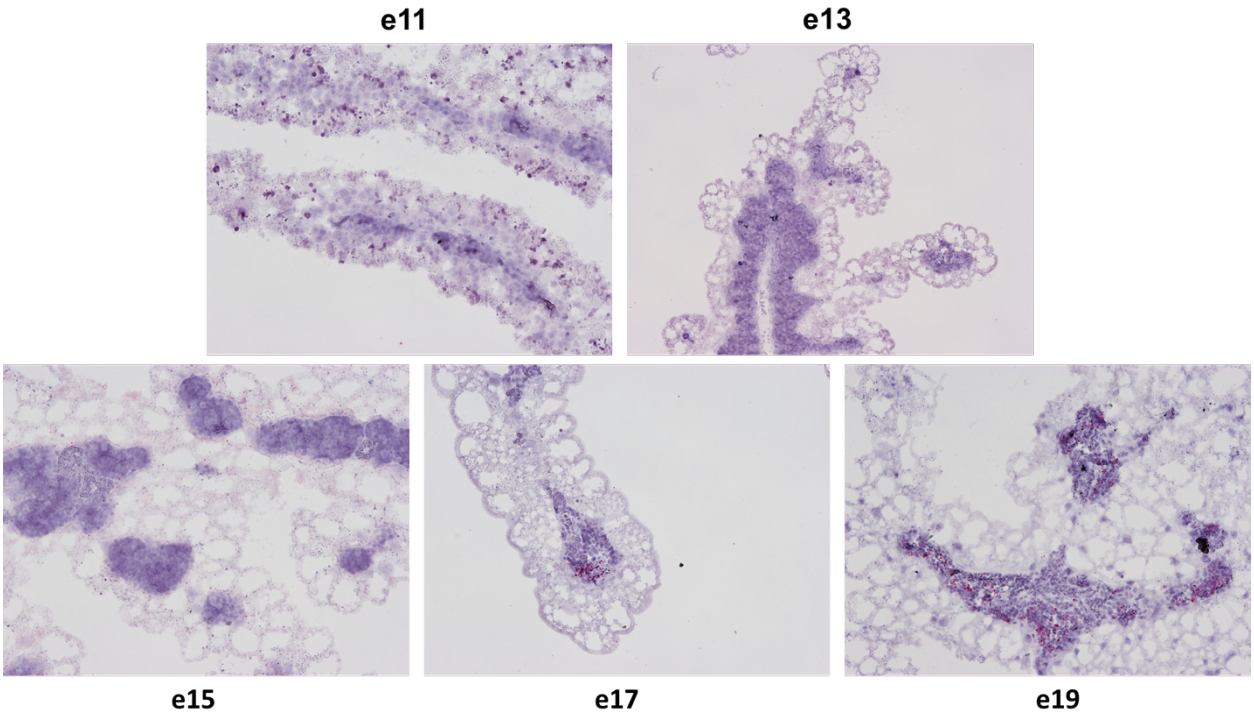

Figure 8.5 In situ hybridization of LEAP2 mRNA in the chicken yolk sac from e11 to e19. The red dots in each picture represent LEAP2 mRNA. The yolk sac samples were counterstained with 50% hematoxylin. All images were captured using 200x magnification.

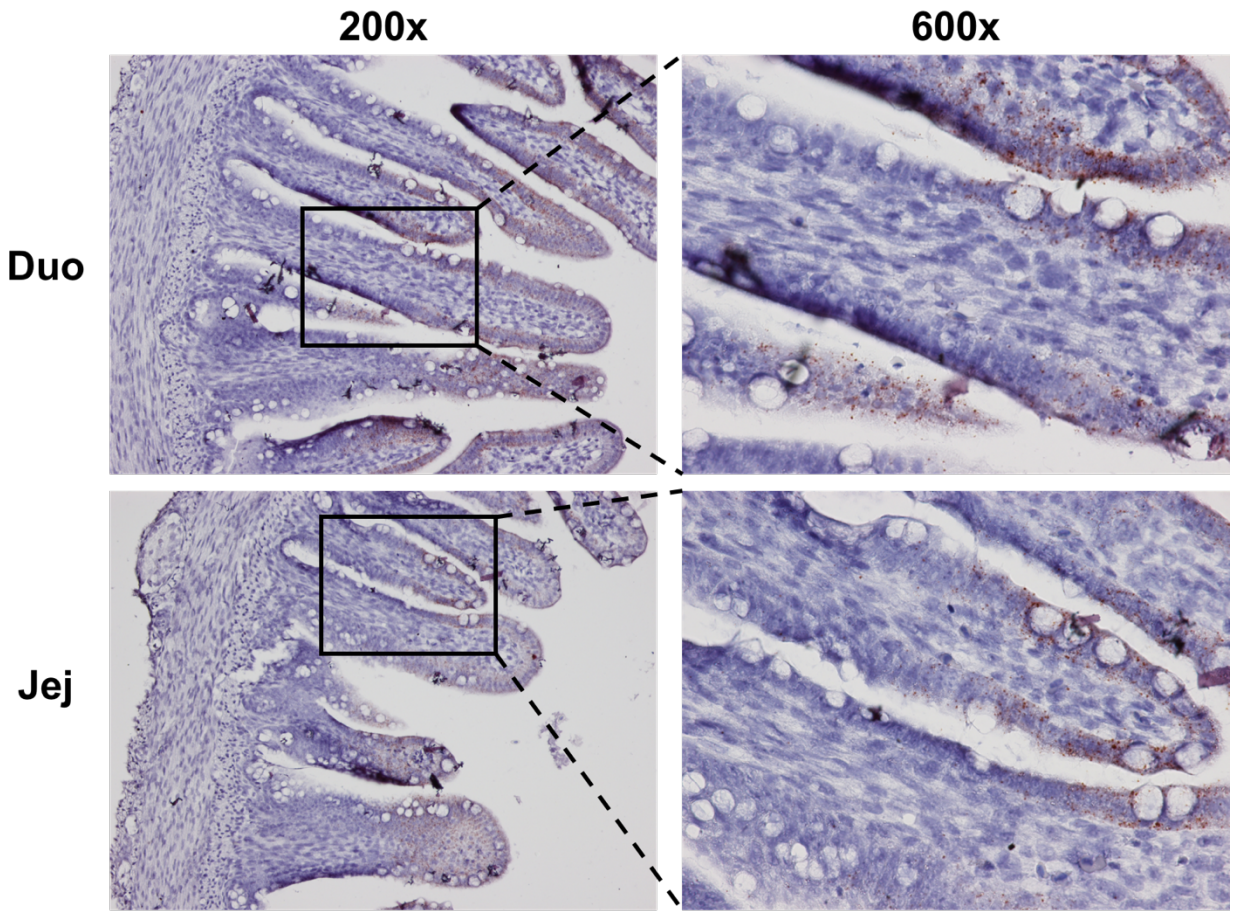

Figure 8.6 In situ hybridization of LEAP2 mRNA in the chicken small intestine (Duo, duodenum; Jej, jejunum) at day of hatch. The brown dots in each picture represent LEAP2 mRNA. The intestinal samples were counterstained with 50% hematoxylin. The 600x magnification images are enlarged from the inserted frames in the 200x magnification images.

Chapter IX

Epilogue

The chicken serves as an excellent model for researching vertebrate embryonic development. Development-associated studies have been elaborately explored for the physiological changes during embryogenesis. The yolk sac is an important extraembryonic tissue, which consists of functional endodermal epithelial cells to help the chicken embryo absorb nutrients. The chicken yolk sac shares many similarities to the small intestine in terms of physiological structure and nutritional function. Both the yolk sac and small intestine form epithelia lined structures with blood vessels distributed at its stroma. These epithelial cells are also able to secrete antimicrobial peptides, digestive enzymes and hormones. Even though many important nutrient transporters such as PepT1 and SGLT1, which are expressed in the chicken intestine, are also found to be expressed in the yolk sac by qPCR, it was still unknown which cell type in either the small intestine or yolk sac was responsible for the nutrient absorptive function.

The standard qPCR technique is not able to distinguish the expression of genes in cells if the tissue samples have heterogeneous cell types. Additionally, the robustness of relative qPCR is highly dependent on the selection of the reference gene or gene combination, which may also be expressed differently in distinct cells. In situ hybridization is a technique to detect DNA or RNA expression in tissues. By combining with histological staining, in situ hybridization can be used to observe the distribution of genes of interest in cells in target tissues. RNAscope *in situ* hybridization is more specific and sensitive than conventional *in situ* hybridization. Each RNAscope chromogen that is detected represents one DNA or RNA molecule, which theoretically makes RNAscope *in situ* hybridization available for quantification through

measuring the intensity of staining. Thus, we performed RNAscope *in situ* hybridization to investigate mRNA expression of PepT1 and SGLT1 in the chicken small intestine.

In mammals and rodents, there are different cell types along the intestinal villus (Figure 2.1). The intestinal stem cells are located in the intestinal crypts with Paneth cells and are able to differentiate into other cell types as they migrate up along the villus. Each type of intestinal epithelial cell expresses particular biomarkers. We used the *in situ* hybridization technique and identified chicken intestinal stem cells expressing Lgr5 and Olfm4 in the intestinal crypts. However, all cells in the chicken intestinal crypts were found to express intestinal stem cell markers, rather than the mammalian model of alternating $Olfm4^{+}/Lgr5^{+}$ stem cells with $Olfm4^{-}/Lgr5^{-}$ Paneth cells. This result suggests two hypotheses: 1. Olfm4 and Lgr5 are chicken intestinal stem cell markers. Thus, chicken intestinal crypts are missing Paneth cells or 2. Chicken intestinal crypts have Paneth cells. Therefore, Olfm4 and Lgr5 are expressed not only in the intestinal stem cells, but also in Paneth cells in chickens. Additionally, we also found that PepT1 mRNA was expressed by enterocytes along the villus and AvBD10 mRNA was expressed by a few cells like enteroendocrine cells up to posthatch d2. However, in the chicken yolk sac, we found that the yolk sac endodermal epithelial cells have multiple functions including nutrient absorption through expressing PepT1 and SGLT1 mRNA and immunity by expressing AvBD10 mRNA. So, different from the intestinal epithelial cells, whose multifunction is due to heterogeneous cell types along the villus, each yolk sac endodermal epithelial cell is able to play multiple roles.

The development of broiler chickens is a process from the embryonic to posthatch stage. The embryonic development determines the posthatch growth and performance of birds. Thus, an understanding of short- and long-term nutritional and immune effects on chicken

embryogenesis would provide strategies to improve the performance of broiler chickens. One of the important ways to supplement nutrients for the chicken embryo is through *in ovo* feeding. *In ovo* supplementation of carbohydrates for chicken embryo increases the body weight of broiler chickens posthatch. Other *in ovo* feeding strategies also produce some benefits for posthatch bird performance. However, the cellular and molecular impact of in ovo feeding on nutrient absorption and embryo development is unknown. Even though many studies have investigated how different diet compositions may change nutrient absorption by altering the expression of nutrient transporters, or change the morphology of the intestinal villus by affecting the villus height or crypt depth, little is known about the cellular change in the small intestine with different kinds and concentrations of feed additives. *In situ* hybridization of different cell markers could provide a better understanding of the shift of different cell types in the target tissues. By using this technique, we may be able to look at the effect of different nutrient supplementation on cell distribution in target tissues and gene expression in specific cell types.